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SEROLOGICAL AND MORPHOLOGICAL STUDIES OF INFECTIOUS CANINE LARYNGOTRACHEITIS VIRUS

by

© Raymond George Marusyk

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Serological and Morphological Studies of Infectious Canine Laryngotracheitis Virus" submitted by Raymond George Marusyk, B.Sc., in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Infectious canine laryngotracheitis (ICL) virus was studied with respect to its serological and morphological characteristics. ICL virus propagated in monolayers of DKL cells was purified and concentrated by CsCl density gradient centrifugation. The buoyant density of purified ICL virus is 1.34 g/ml and the UV absorption ratio 260:280 is 1.18. The infectivity and haemagglutinating properties of ICL virus are associated with, and are inseparable from, the intact virus particle. Sedimentation to a pellet, treatment with fluorocarbon, and sonication for more than 30 seconds were found to inactivate viral infectivity and haemagglutination.

Haemagglutination experiments with a variety of animal erythrocytes have shown the ICL virus haemagglutinin to be specific for human erythrocytes. The haemagglutinin was adsorbed to human 'O' RBC at 37° C, but could not be eluted at 4° C. Trypsin treatment at 37° C for 1 hour did not inactivate the haemagglutinin.

DKL cells used for virus propagation, could not be used for assay of virus infectivity as the cells did not remain viable, however plaque formation of ICL virus on MDC cells was possible and was enhanced by the addition of HLA to the overlay medium. DEAE-dextran, protamine sulfate, and DMSO added to the overlay medium also had an effect on the size and number of plaques formed. Particle counts of ICL virus showed that one infectious unit (PFU) is equivalent to approximately 5,000 virus particles.

The presence and relationship of three ICL virus antigens was shown by agar gel double diffusion studies. Immuno- and electrophoretic studies of degraded ICL virus showed that the virus protein was negatively charged at pH 7.0 - 8.6.

Electron microscopic examination of intact and degraded ICL virus revealed that it is a typical adenovirus. The virus capsomere consists of three subunits: the hexon, the penton, and the fibre which are eluted from a DEAE-cellulose column at 0.05 M, 0.2 M, and 0.3 M NaCl respectively. The fibre subunit, located at each vertex of the intact virus, is 35 mμ long. Comparison of the size of the intact ICL virus with 88 mμ latex particles shows that the virus is approximately 80 mμ in diameter.

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TO HALYNA

-- Do not become a mere
recorder of facts, but
try to penetrate the mystery
of their origin --

Anon

TABLE OF CONTENTS

Page

MATERIALS AND METHODS - Continued

LIST OF TABLES

LIST OF FIGURES

Figure No.		Facing Page

ABBREVIATIONS

ICL	- infectious canine laryngotracheitis
ICH	- infectious canine hepatitis
HCC	- *Hepatitis contageosum canis*
MEM	- minimal essential medium
HLA	- Hank's lactalbumin medium
DKL	- dog kidney line
MDC	- Mabin - Darby canine kidney
RBC	- red blood cell(s)
HA	- haemagglutination
HAI	- haemagglutination - inhibition
HAn	- haemagglutinin
PBS	- phosphate buffered saline
DEAE	- diethylaminoethyl
DMSO	- dimethyl sulfoxide
Tris	- tris (hydroxymethyl) - aminomethane
PFU	- plaque forming unit
$TCID_{50}$	- 50 % tissue culture infectious does
rpm	- revolutions per minute

All temperatures are given in Centigrade degrees.

INTRODUCTION

The canine adenovirus, previously designated as Toronto A26/61, but now known as infectious canine laryngotracheitis (ICL) virus has been studied and partially characterized by Ditchfield _et al_ (1962), Yamamoto (1963, 1966, 1967), and Gaunt (1966). The virus has been shown to be an adenovirus producing characteristic cytopathic effect (CPE) with intranuclear inclusions and sharing common complement-fixing antigens with canine hepatitis virus and with the human adenovirus type 3. ICL virus was shown to be related to infectious canine hepatitis (ICH) virus on the basis of tissue culture susceptibility, complement fixation, neutralization, and haemagglutination-inhibition tests.

The first members of the adenovirus group were reported in 1953 (Rowe _et al_, 1953). Since then, discovery of new members has resulted in the current recognition of at least forty-four serotypes (Pereira _et al_, 1963), which are divided into human, simian, bovine, canine, murine, and avian adenovirus subgroups. The criteria for inclusion (Pereira _et al_, 1963) in this grouping are:

a) the virus contains deoxyribonucleic acid;

b) the virus diameter is estimated by various techniques as 60 - 85 mμ;

c) the virus is resistant to ether treatment, is very stable at low temperatures but readily inactivated at 56° C, and is stable over a wide range of pH;

d) the virus has an outer covering (capsid) apparently icosahedral (5-3-2 symmetry) formed by 252 subunits called capsomeres (there is no pericapsidal membrane);

e) the virus multiplies in the nucleus of infected cells and induces the formation of a specific protein which in turn causes characteristic toxicity in the cell culture;

f) the virus, with the exception of avian adenovirus, shares a group-specific antigen with other members of the group and which is detectable by complement fixation and gel diffusion tests.

In 1958, Rosen first reported that the human adenoviruses were capable of haemagglutination, and in 1960, he reported that the haemagglutination-inhibition test could be used to serologically identify most of the known human adenovirus serotypes previously identified on the basis of the neutralization test. Rosen divided the viruses into four groups on the basis of their haemagglutination characteristics with erythrocytes from different animals.

Human adenoviruses belonging to group 1, agglutinate rhesus monkey erythrocytes, group 2 agglutinate rat erythrocytes, and group 3 partially agglutinate rat erythrocytes. Serotype 18 has not been reported to agglutinate erythrocytes. Rosen's original classification of the 18 serotypes, has been extended to include the 31 human serotypes currently known (Rosen _et_ _al_, 1962; Pereira _et_ _al_, 1965).

ICH virus, a non-human adenovirus serotype, was reported to agglutinate human 'O' and guinea pig erythrocytes (Kunishige and Hirato, 1960), but not those from sheep, rabbits, fowl, horses, cows,

dogs or mice. A subsequent report by Espmark and Salenstedt (1961) stated that ICH virus will also agglutinate rat erythrocytes.

Simon (1962a) on investigating the haemagglutinins of human adenovirus serotypes 3 and 7, found that after adsorption to rhesus erythrocytes at 37° C, elution of the haemagglutinin from the erythrocytes occurred when the erythrocyte-haemagglutinin complex was incubated at 4° C. Serotypes 11 and 16, although adsorbed at 37° C, were not eluted at 4° C. Although Simon found that 0.25 % trypsin had no effect on the type 3 agglutinin when incubated at 37° C for one hour, Bauer and Wigand (1962) reported that type 11 haemagglutinin, which belongs to the same group, was reduced in titre when exposed to 1 % trypsin for one hour. Buckland and Tyrell (1963) reported that papain at 1 % concentration and bisulphite at 0.01 M did not inactivate the type 7 haemagglutinin. Receptor-destroying enzyme (RDE; from Vibrio cholerae), at concentrations that destroy receptors for influenza virus, had no effect on the erythrocyte receptors for the group 1 adenoviruses (Simon 1962a). Simon also reported that trypsin and periodate decreased the susceptibility of rhesus erythrocytes to agglutination by type 3 adenovirus.

Buckland and Tyrell (1963) reported that RDE reduced the susceptibility of rhesus erythrocytes to agglutination by type 7 adenovirus. Periodate (0.0058 M) and papain (2 %) also reduced agglutinability by type 7 appreciably, while chymotrypsin (0.01 %) produced only a small reduction. These studies on the haemagglutinin and the erythrocyte receptors indicate that they are protein in nature.

A number of non-infectious, but virus specific, antigens have been found in adenovirus-infected tissue culture fluids in addition to infectious virus (Wilcox and Ginsberg, 1961). The soluble particles found in virus-infected tissue culture fluids seem to be normal virus components produced in excess. The group specific and type specific complement fixing antigens are produced even when the synthesis of infectious virus has been decreased by the incorporation of proflavine in the medium (Wilcox and Ginsberg, 1961). The antigens are synthesized two to three hours after synthesis of DNA begins and two hours before the appearance of infectious virus (Flanagan and Ginsberg, 1962). On the basis of the studies of Ginsberg and his colleagues, who investigated adenovirus types 4 and 5, the evidence would indicate that the adenovirus soluble antigens are normal non-assembled virus components synthesized in excess and released from the cells.

Pereira et al (1959a) found that, by submitting partially purified adenovirus type 5 preparations to agar gel diffusion and immunoelectrophoresis, they were able to separate three non-infectious antigens which they labelled A, B, and C. Wilcox and Ginsberg (1961) confirmed the existence of the three antigens, which they labelled L, T, and E corresponding to A, B, and C respectively. They were able to separate the antigens by DEAE-cellulose column chromatography as well as by gel-diffusion.

To avoid ambiguity and subsequent confusion introduced by the different terms previously employed for the same antigens, Ginsberg et al (1966) proposed a terminology based on the morphological characteristics

of the capsid subunits and the corresponding soluble antigens (Norrby, 1966; Wilcox and Ginsberg, 1963; and Valentine and Pereira, 1965).

"1. It is recommended that the major subunits, of which there are 240 per virion, be called the hexon to denote that each unit has 6 neighbours. Hence, it is proposed that the corresponding soluble antigen (A or L) be termed the hexon antigen.

2. The unit at the 12 corners of the icosahedron should then be termed a penton because each has 5 neighbouring units (hexons). The penton corresponds to the B antigen, thus, the soluble antigen should be called the penton antigen.

3. The penton is composed of a base and a fibre. It is proposed that the C (E) antigen be known as the fibre antigen."

The fractionation of these components on DEAE-cellulose (Wilcox and Ginsberg, 1961; Klemperer and Pereira, 1959; and Valentine and Pereira, 1965) revealed that each was eluted at a characteristic molarity of NaCl: fibres at 0.05 M, pentons at 0.2 M, and hexons at 0.3 M NaCl. The three types of particles recovered are not homogeneous antigenically. On gel diffusion, spur formation of the precipitin lines demonstrates a reaction of partial identity between the penton and fibre antigens (Klemperer and Pereira, 1959; Allison et al, 1960; and Valentine and Pereira, 1965). The hexon and fibre antigens do not demonstrate any reactions of identity.

Wilcox and Ginsberg (1963) and Valentine and Pereira (1965) found, on electron microscopic examination of antigen preparations purified by chromatography on DEAE-cellulose, that the hexon antigens

were morphologically similar to, or identical with, the capsomeres of the intact virus. The electronphotomicrographs of Valentine and Pereira (1965) revealed the fine structure of the hexon, penton and fibre subunits. The hexon, of adenovirus type 5, is a round object 8 mμ in diameter. The penton has a round head 8 mμ in diameter attached to a tail 20 mμ long and 2 mμ wide with a 4 mμ knob on the end. The fibre resembles the penton without its head. The molecular weight of the subunits ranges from 70,000 to 280,000.

When adenovirus type 3 or 7 was mixed with an erythrocyte suspension, the infectious virus particles and the haemagglutinins were adsorbed to the erythrocytes (Simon, 1962a; Zuschek, 1961; Norrby, 1966). On high speed centrifugation, Zuschek (1961) found that the infectious particle sedimented faster than the haemagglutinin, which in turn sedimented faster than the complement fixing antigen. Bauer and Wigand (1962) found that on high speed centrifugation of an adenovirus type 11 suspension, they were able to separate a part of the haemagglutinating activity from the infectivity.

Norrby et al (1964) and Norrby (1966) have shown that the haemagglutinin of adenovirus type 3 is completely separable from the intact virus particle and is composed of aggregates of the fibre subunit arranged in a 'rosette-like' structure. Norrby and Wadell (1967), working with adenovirus type 4, have separated two non-virus associated haemagglutinins from virus-infected tissue culture suspensions. They were able to show that these haemagglutinins were identical to viral subunits.

The evidence above, would indicate that the haemagglutinin, of at least some adenoviruses, can exist as a separate entity from the infectious particle.

Norrby et al (1964) reported that concentration of adenovirus type 3 by centrifugation to a pellet caused a considerable loss of infectivity. Also, the studies by Gaunt (1966) with ICL virus showed a similar inactivation of infectivity and haemagglutination. In order to prevent the loss of infectivity and haemagglutination, Norrby et al (1964) utilized a CsCl density gradient centrifugation for virus purification. They showed that sedimentation of the adenovirus onto a CsCl layer, would separate the virus from cell protein with a higher recovery of infectivity and haemagglutinin.

The assay of adenovirus infectivity, by the plaque assay method of Dulbecco (1952), is a difficult procedure. Long incubation periods, often 10 to 21 days, are required for maximum detectable changes to develop in infected cells, and, therefore, special technical problems must be solved for each assay system employed. The maintenance of a viable cell monolayer is dependent upon adequate nutritional requirements and pH. Bonifas and Schlesinger (1959) and Kjellen (1961) in studying adenovirus-host cell systems by plaque assay, had to formulate specific overlay media which would satisfy the requirements of the system. Rouse et al (1963) found that cell lines used for plaque assay were often contaminated with pleuro-pneumonia-like organisms (PPLO). The PPLO rapidly deplete the available arginine in the overlay medium, which in turn results in loss of cell viability and inhibition of virus plaque formation.

Sulfated polysaccharides present in agar (Araki, 1959) have been found to possess inhibitory activity for certain viruses (Takemoto and Liebhaber, 1961; Colter et al, 1964) and the addition of DEAE-dextran or protamine sulfate to the agar to bind these substances (Liebhaber and Takemoto, 1961; Miles and Austin, 1963; Colter et al, 1964; Campbell and Colter, 1965) has been recommended for work with certain viral agents.

This study was undertaken to characterize and purify ICL virus and to compare the haemagglutinating property, morphology, and antigenic nature with that of the human adenovirus serotypes.

MATERIALS AND METHODS

1. Virus Strains

The canine adenovirus previously designated as Toronto A26/61 (Ditchfield, 1962), but redesignated as infectious canine laryngotracheitis (ICL) virus (Yamamoto, 1966), was obtained from Dr. J. Ditchfield, University of Toronto. The virus has been continuously propagated in a cell line (DKL) derived from dog kidney tissue culture.

The infectious canine hepatitis virus (ICH) was obtained from Dr. V. J. Cabasso, Lederle Laboratories, Pearl River, New York. The second strain of infectious canine hepatitis virus (*Hepatitis contageosum canis* - HCC) was obtained from Dr. J. G. Kapsenberg, Rijks Institut Voor de Volksgezonheid Sterrenbos, 1 Utrecht, Netherlands. Frozen stocks of these virus strains were maintained at -70° C.

2. Tissue Culture Cells

Two lines of dog kidney cells were employed in this study, *viz*., a continuous epithelial dog kidney cell line (DKL) used for ICL virus production and the Mabin - Darby Canine kidney cell line (MDC) used for assay of viral infectivity. The DKL cell line was obtained from Dr. R. C. Parker, Connaught Medical Research Laboratories, University of Toronto, and the MDC cell line was obtained from the American Type Culture Collection (ATCC).

3. Tissue Culture Media

Two types of media were used throughout this study:

a) Minimal essential medium - Eagle base (MEM) is a powdered medium supplied by General Biochemicals, Grand Island, New

York. No information as to the method of formulation has been released. Greene et al (1965) did however, report on the use of this medium for maintenance of tissue culture. MEM supplemented with 2.5 % calf serum adjusted to pH 7.4 with 7.5 % sodium bicarbonate and containing 100 IU/ml of penicillin-G and 100 μg of streptomycin-sulfate/ml, was used for propagation of DKL and MDC.

b) Hanks' lactalbumin medium (HLA) was composed of Hanks' balanced salt solution (HBSS) (Hanks and Wallace, 1949) with 0.5 % lactalbumin hydrolysate (DIFCO 0996 - 01). pH adjusted HLA containing antibiotics was prepared for use in the plaque assay system developed in this study.

4. Tissue Culture Maintenance

DKL and MDC cell lines were normally maintained in Roux bottles. Bottles containing complete monolayers were washed and treated with 5×10^{-4} M EDTA in HBSS at 37° C for a sufficient time to detach the cells from the glass. The cell suspension was centrifuged at 800 rpm for 10 minutes. The supernatant was removed and the cell pellet resuspended in sufficient growth medium so that each bottle to be seeded received an aliquot of 125 ml of cell suspension at a cell concentration of approximately $2\text{-}3 \times 10^5$ cells per ml. A complete monolayer was usually formed in three to four days and the cells were a) infected with virus, b) used as control cultures, or c) incubated at 37° C until conditions required re-seeding.

5. Virus Production

Twelve Roux bottles containing complete monolayers of DKL cells were routinely used for propagation of ICL virus. Prior to infection of the monolayer, the tissue culture medium was removed and the cells were washed once with fresh medium. Twenty ml of a virus suspension (1.0 - 2.0 x 10^6 PFU/ml) were added to the cell monolayer. The infected monolayers were then incubated at 37°C for 90 minutes with the bottles being gently rotated at fifteen minute intervals. After the 90 minute adsorption time, 125 ml of fresh tissue culture medium were added. The infected monolayers were incubated for 5 days at 37° C. After complete cytopathic effect had occurred (by the end of the five day incubation), the infected cell cultures were subjected to three cycles of freeze-thawing (-70° C and room temperature) and pooled. The pooled tissue culture harvest was then centrifuged at 8,000 rpm for 15 minutes in a Spinco #19 rotor to remove the cellular debris. The supernatant remaining was called the crude virus suspension.

6. Virus Assay

Two methods of assaying for viral infectivity were utilized:

a) Plaque assays for ICL virus infectivity were carried out using a modification of the technique described by Dulbecco (1952). MDC cells (2 - 3 x 10^5 cells/ml) for plaque assay were seeded onto disposable plastic tissue culture dishes (6 cm diameter) and maintained in MEM tissue culture medium supplemented with 5 % calf serum, adjusted to pH 7.4 with 7.5 % sodium bicarbonate, and containing 200 IU/ml of penicillin-G

and 200 μg/ml of streptomycin sulfate. As the remainder of the plaque assay procedure was developed in this study, a description of the procedure used is in the Results section.

b) The 50 % tissue culture infectious dose ($TCID_{50}$) of ICL virus was determined in tube cultures of DKL cells. The cells were maintained in the same medium described above until required for infectivity assay. Serial ten-fold dilutions of the virus were prepared in HBSS and, from each dilution, four tube cultures were inoculated with 0.1 ml each. Examination of the cell monolayers for CPE was made daily for 14 days after infection, and the log $TCID_{50}$ was calculated by the Reed and Muench (1938) method.

7. Erythrocytes

Human group 'O' erythrocytes (RBC) were normally employed for haemagglutination (HA) assays. The RBC were washed three times in physiological saline and the packed cell volume determined by centrifugation at 1,500 rpm for 20 minutes. The cells were resuspended to the required concentration in phosphate buffered saline (PBS; Dulbecco and Vogt, 1954), physiological saline, or Alsever's solution (Kalter, 1963). RBC suspended in saline were used for HA assay while RBC suspended in PBS were utilized in pH stability experiments. RBC were stored in Alsever's solution at 4° C for periods of up to two weeks. After storage, the RBC were washed twice before use. Rosen (1960) claims that there is no loss in agglutinability with such storage. Other RBC

from various animal species used in this study were prepared in a similar manner.

8. Haemagglutinin and Haemagglutination-Inhibition Assays

Serial two-fold dilutions of virus were prepared in 0.5 ml volumes in physiological saline in 85 mm x 10 mm tubes. To each tube was added 0.25 ml of a 0.5 % suspension of RBC in saline. Tubes were shaken and incubated at room temperature for two hours. Haemagglutination in the tubes was determined from the pattern of settled cells (see Fig. 2). The haemagglutinin titre was taken as the highest dilution of virus, prior to addition of the RBC suspension, which produced complete agglutination of the RBC. The settling patterns were recorded as + (complete agglutination), P (partial agglutination), or - (no agglutination).

This standard haemagglutination technique used for diagnostic work, is quantitatively inaccurate as the same pipette is used throughout the dilution series. In order to accurately determine the HA titre of virus preparations for particle count calculations, a standard curve (Fig. 1) of HA titres was prepared as follows: HA titres of virus suspensions determined in the manner described above were recorded as experimental values. HA titres of the same virus suspensions diluted with a clean pipette for each dilution were recorded as corrected values. The curve shown in Fig. 1 was prepared. Corrected HA titres for experimental HA assays were then determined from this curve.

The haemagglutination-inhibition (HAI) titre of virus antiserum was determined by making serial two-fold dilutions of the antiserum in 0.5 ml volumes of physiological saline. 0.25 ml of a 0.5 % suspension

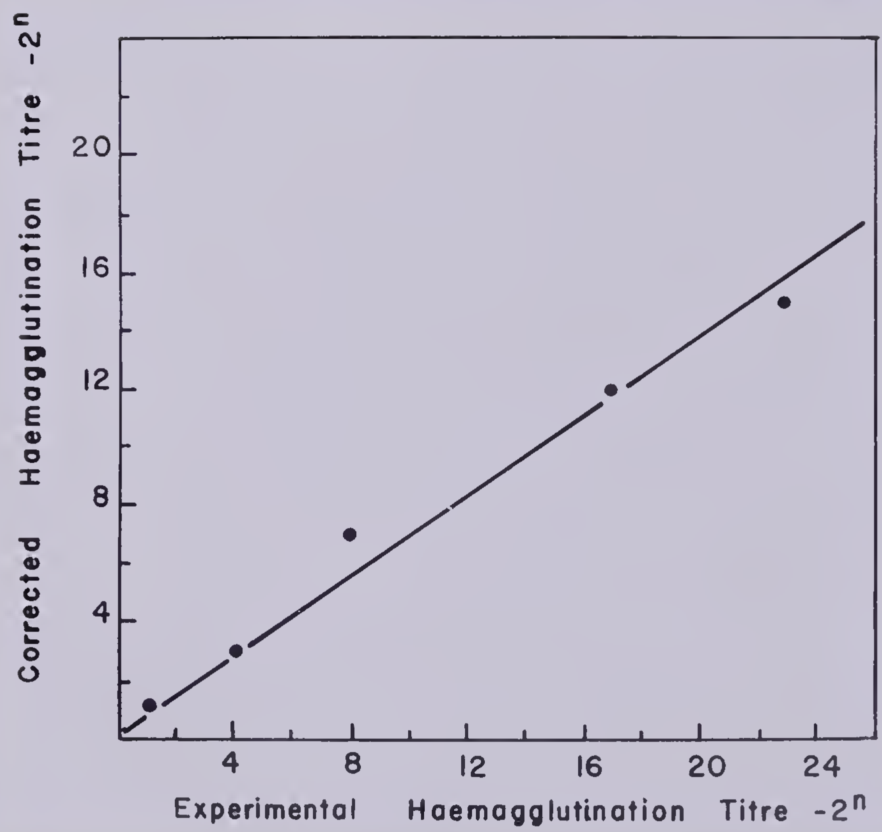

Corrected Haemagglutination Titre -2^n
20
16
12
8
4
4
8
12
16
20
24
Experimental Haemagglutination Titre -2^n

Fig. 1

Correction Curve for HA Tests

HA titres of virus suspensions determined in the manner described in Materials and Methods are recorded as experimental values. HA titres of the same virus suspensions, in which each dilution was prepared with a different pipette, are recorded as corrected values.

of RBC and 0.25 ml of a virus suspension of a known HA titre were added to each tube. The racks were shaken and incubated at room temperature for two hours. Readings were made according to the pattern of settled cells. The HAI titre was taken as the highest dilution of antiserum which would completely inhibit agglutination of the RBC.

9. Sonic Oscillation

Sonic oscillation (sonication) of virus suspension were carried out with a Bronwill Biosonik (Bronwill Scientific, Rochester, N.Y.). Thirty ml of the virus suspension to be sonicated were placed in a heavy-walled 60 ml centrifuge tube and covered with a plastic cap which had a 1/4 inch diameter hole at the top. The sonicator probe was immersed in the virus suspensions to a depth of 1/4 inch. Sonication was carried out at maximum power (setting 100) for 30 seconds. For periods longer than 30 seconds, the centrifuge tube was cooled in an ice-alcohol bath.

10. Negative Pressure Dialysis

Virus concentration by negative pressure dialysis was carried out with a S & S Collodion Bag Apparatus (Carl Schleicher and Schuell Co., Keene, N.H.). The apparatus was assembled as described by the manufacturer, the virus suspension introduced, and a vacuum of 500 mm Hg applied to the suction vessel. The entire apparatus was cooled in an ice bath. A ten-fold concentration of virus material could be achieved in two hours.

11. Buoyant Density Determination

The buoyant densities in CsCl of fractions collected after CsCl

density gradient centrifugation were determined by weighing 20 μl aliquots on a Mettler Type H6T balance. An equal volume of ion-exchange water was also weighed and the buoyant density for each fraction computed.

12. Optical Density Measurements

Optical density measurements at 260 mμ and 280 mμ of 1 ml aliquots of virus or virus protein suspensions were routinely determined in one of the three following instruments: a) Gilford Model 2000 Automatic Spectrophotometer (Gilford Inst. Labs. Inc., Oberlin, Ohio), b) Beckman DB-G grating spectrophotometer (Beckman Instr., Palo Alto, California), c) Bausch and Lomb Model '505' recording spectrophotometer (Bausch and Lomb Co. Ltd., Toronto, Ontario).

13. Column Chromatography and Gel Filtration

a) Thoroughly washed DEAE-cellulose (Eastman Organic Chemicals Co., Rochester, N.Y.) (Elbart and Sober, 1962) was mixed with 0.01 M Tris-HCl buffer, pH 7.2 to give a 20 % suspension. This suspension was stored until required. For preparation of chromatography columns, the suspension was diluted with 0.01 M Tris-HCl buffer, pH 7.2, and added to a 1.3 cm diameter column to a height of 15 cm (or as desired). The column was then washed with several volumes of buffer.

b) Bio-Gel A15m, (BioRad Laboratories, Richmond, California) available in slurry form, was added to a 1.3 cm diameter column to a height of 30 cm in the manner recommended by the supplier.

The column was then washed and equilibrated with several volumes of 0.01 M Tris-HCl buffer, pH 7.2.

14. Agar Gel Double Diffusion and Agar Gel Electrophoresis

a) Agar gel double diffusion plates were prepared by adding 10 ml of melted 1 %, 1.5 % or 2 % Noble agar in ion-exchange water, to 6 cm diameter disposable tissue culture dishes. 0.5 % sodium azide was added as a preservative. After the agar had solidified, circular wells 7 mm in diameter (1.5 cm center to center inter-well distance) were cut with a brass cork borer: one in the center and 5 in a circle equidistant to it. Each well was 'sealed' with a droplet of melted agar. The plates were then stored at 4° C until used.

(b) Agarose (L'industrie Biologique Francaise S.A., Gennevilliers, France) coated microscope slides for agar gel immunoelectrophoresis were prepared in the manner of Parker _et al_ (1962). 4 ml of melted 1 % agarose in the electrophoresis buffer were delivered on to silicon-coated microscope slides placed on a level surface. 0.5 % sodium azide was added as a preservative. After the agarose had solidified, 5 mm diameter reservoir wells were cut (see Fig. 27) with a brass cork borer. For immunoelectrophoresis, 2 mm wide antiserum wells were cut by means of two Gem razor blades taped together. The prepared slides were stored at 4° C in petri plates containing moist filter paper.

c) Electrophoresis of samples on agarose coated slides was carried out in a Gelman Deluxe Electrophoresis Chamber (Gelman Inst. Co., Ann Arbor, Mich.). The slides were supported on plexiglass racks within the chamber, and connected to the electrode baths by means of white blotting-paper wicks cut the same width as the slide. Several sheets of blotting paper were attached to the inside of the chamber immediately above the slides in order to prevent excess moisture from falling on the slide. Electrical power was supplied by a Metaloglass electrophoresis power unit (Metaloglass, Inc., Boston, Mass.) or a Millipore PhoroSlide power source (Millipore Ltd., Montreal, P.Q.).

d) For immunoelectrophoresis, the test antigens were allowed to react with the antiserum in a sealed humidity chamber at room temperature for 48 hours. The slides were then washed in physiological saline in order to remove excess antigen or antibody (at least 300 ml saline per slide for 24 hours). The agarose surface was then covered with moist filter paper and placed in a 37° C incubator for 6 to 8 hours. After incubation, the salts and water were drawn into the filter paper, leaving a paper-thin, clear agarose film adherent to the slide surface. The slides were then immersed in the following stain (Uriel, 1964) for at least three hours: nigrosine - 1 g; acetic acid 1 M - 450 ml; sodium acetate 0.1 M - 450 ml; glycerine - 100 ml.

After staining, the agarose films were clarified with several volumes of 2 % acetic acid and dried for 15 minutes at 37° C.

e) Agarose slides for electrophoresis (Uriel, 1964) were removed from the chamber and immersed in a fixing solution of 2 % acetic acid for 3 hours or 20 % sulfosalicylic acid for 10 minutes. Agarose slides fixed in sulfosalicylic acid were often damaged as the acid appeared to effect the constitution of the gel. The agarose slides were then stained and dried as described above.

f) After the final drying, the agarose films were covered with another glass slide, sealed, and photographed by placing of the slide on a sheet of Kodak contact paper and exposing the paper with a bright light located directly overhead.

15. Electron Microscopy

Electron microscopic examination of virus preparations was carried out on a Philips EM200 electron microscope operated with a 60-kv beam and double condenser illumination.

a) Copper (150 mesh) or gold (200 mesh) grids (E. F. Fullam, Inc., Schenectady, N.Y.) were covered with a film of formvar and where necessary, stabilized with evaporated carbon.

b) Negative staining of virus preparations was carried out according to the method of Horne et al (1959). Solutions of 3 % sodium silicotungstic acid (SSTA) and 3 % phospho-tungstic acid (PTA) were adjusted to pH 6.8 with KOH.

c) Virus preparations were shadowed with palladium and stabilized with evaporated carbon in an EFFA vacuum evaporator (E.F. Fullam Inc., Schenectady, N.Y.).

d) Concentrations of polystyrene latex particles (Dow Chemical Co., Midland, Michigan) averaging 0.088 μ and 0.264 μ in diameter were determined from dry-weight measurements and from data supplied by the manufacturer. The latex suspensions were stored at a concentration of approximately $3 - 4 \times 10^{10}$ particles per ml in glass-distilled water.

e) Particle counts of virus and latex suspensions were performed by the lowered drop method in the manner of Pinteric and Taylor (1962). 0.1 ml of a calibrated suspension of latex particles was mixed with an equal volume of virus suspension, and several drops of the mixture placed on top of a formvar film floating on the surface of an ammonium acetate-ammonium carbonate buffer (pH 7.3 - 7.6). The drops were allowed to dialyze for several hours. The level of the buffer was then lowered until the drops were centred on gold grids resting on a piece of sintered glass. The grids were then dried in a covered petri dish containing pellets of NaOH. The grids were shadowed as described above and examined in the electron microscope. Counts of latex and virus particles were facilitated by placing the electronphotomicrograph negative in a photographic enlarger and projecting the image onto a large sheet of white paper.

16. Buffer Solutions

Buffer solutions required for this research project were prepared from tables listed by Gomori (1955).

EXPERIMENTAL PROCEDURES AND RESULTS

Relation of ICL virus to Other Canine Adenovirus Strains

ICL virus and the two strains of infectious canine hepatitis virus (ICH, HCC) were examined by the haemagglutination-inhibition (HAI) test in order to determine their serological relationship. HAI tests were carried out with stock virus suspensions and with antisera (prepared by intravenous injection of the specific virus into rabbits) against each of the viruses. Table I summarizes the results of the experiments performed.

TABLE I

Virus Tested	Haemagglutination-inhibition Titre ICL antisera	ICH antisera	HCC antisera
ICL	1024*	512	128
ICH	512	4096	1024
HCC	1024	1024	1024

* Reciprocal Haemagglutination-inhibition titre - a haemagglutination inhibition unit is defined as the highest dilution of antisera in a 0.5 ml volume causing complete inhibition of haemagglutination (HA) of RBC by the particular virus added to the dilution system.

The results indicate that there is a serological relationship between the three canine adenovirus strains as shown by the cross reaction between each of the strains and the antisera of the other two strains.

Standard Deviation of ICL Virus Haemagglutinating Activity (HA)

For the correct evaluation of the results obtained in ICL virus HA assays, 30 parallel HA titrations were performed in the manner described in Materials and Methods. The standard deviation was calculated from the logarithms of the HA titres. Table II shows that the standard deviation of the HA assays with ICL virus was 0.16 log units for the 30 parallel experiments.

TABLE II

Virus	No. of Assays	Log Mean HA Titre	Standard Deviation
ICL	30	1.89	0.16 log units*

* The standard deviation was calculated according to the method of Spiegel (1961).

The results obtained would indicate that if 4 parallel experiments were conducted, a 3.0 (antilog 0.48 = 3 x SD) titre difference would be significant. This would incorporate a one tube variation in a particular twofold dilution series.

Erythrocyte Specificity of ICL Virus Haemagglutinin (HAn)

Previous studies with ICL virus in this laboratory (Gaunt, 1966) showed that ICL virus will agglutinate all four groups of human erythrocytes (RBC). As ICL virus has been shown to be serologically related to ICH virus, which reportedly agglutinates chicken RBC (Fastier, 1957), experiments were carried out with numerous species of RBC in order to determine if ICL virus would agglutinate any other than the human variety. The various species of RBC used in the experiment were prepared as described in Materials and Methods. In order to determine if temperature and time were variables in the HA system, the assays were carried out at 4° C, room temperature, and 37° C. The results were recorded at 30 minutes, 120 minutes, and 1200 minutes. Controls consisted of physiological saline and an equal amount of a 0.5 % suspension of the indicated RBC.

The results obtained indicate that ICL virus will yield positive agglutination of human 'O' RBC. Fig. 2 shows the various types of agglutination patterns obtained. Of the other species of RBC tested (murine, hamster, goose, rabbit, sheep, canine, rhesus, rat and chicken),

Fig. 2

<u>Settling Patterns in Haemagglutination Titrations</u>

The manner in which RBC settle indicate the relative amount of agglutination.

Upper row (left to right): prozone, prozone, positive positive, positive, positive, negative, negative, negative.

Lower row: prozone, prozone, partial, partial, positive, positive, positive, negative, negative.

only rabbit and rat RBC reacted to any degree with ICL virus. Temperature and time of incubation did not appear to have a great effect on the HA assays. The majority of assays were readable after two hours incubation at room temperature. However, some RBC did settle faster at all temperatures.

Rabbit RBC gave partial reactions over a wide range of virus dilutions rather than the usual positive agglutination obtained with human 'O' RBC. Repeated experiments with rabbit RBC and ICL virus carried out at different pH's (from 5.0 to 9.0) did not change the settling patterns obtained.

Rat RBC also gave a characteristic partial agglutination pattern. Human adenoviruses of group 3 are typed according to their ability to partially agglutinate rat RBC (Rosen, 1960). In order to bring about a complete positive agglutination of the RBC, a heterologous virus antiserum has to be added to the assay system. To ascertain if such a heterologous antisera system was required for the complete agglutination of rat RBC with ICL virus, antisera against ICH, HCC, human adenovirus types 1, 2, 6, 7 and 12 were utilized in different HA assays. None of the above antisera tested gave results different to that previously attained, indicating that a heterologous antiserum had no effect on the partial agglutination pattern of rat RBC by ICL virus.

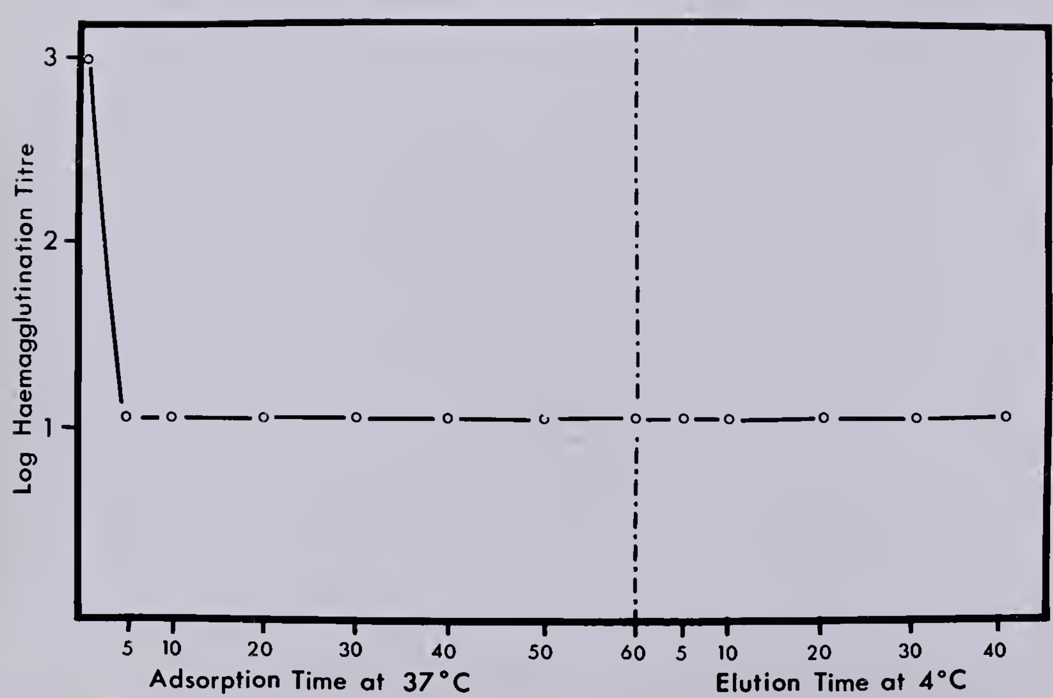
<u>Adsorption and Elution of ICL Virus to Human Erythrocytes</u>

A study of the adsorption of ICL virus to whole RBC was conducted according to the method of Simon (1962a). 1.5 ml of washed human 'O' RBC were added to 30 ml of virus suspension (HA titre 128) at 37° C in a water bath. At varying time intervals, aliquots of the virus-RBC

suspension were removed and centrifuged (Spinco Microfuge, 18,000 xg for 1 minute) to precipitate the RBC and attached virus. The supernatant was assayed for HA activity. The experiment shown in Fig. 3 illustrates that ICL virus is adsorbed very rapidly to the RBC but after five minutes, no further adsorption appears to take place.

Since certain human adenoviruses are known to elute from RBC at low temperatures (Simon, 1962a), the virus-RBC was examined for instability at 4° C. The precipitates from the adsorption study were diluted to the original volume with saline at 4° C and the mixture kept in an ice bath. Samples were removed at varying time intervals, centrifuged, and the supernatant assayed for HA. This result is also shown in Fig. 3. The ICL virus haemagglutinin could not be eluted over the time intervals studied. Further studies indicated that no elution occurred at room temperature or at 37° C, demonstrating that the virus-RBC complex does not dissociate.

Visualization of the attachment of ICL virus to RBC stroma was attempted. The stroma were prepared by suspending washed RBC in distilled water until haemolysis took place. The stroma were centrifuged to a pellet in a Spinco Microfuge (18,000 xg for 1 minute) and washed with several changes of distilled water to remove any residual haemoglobin. The white precipitate was resuspended in 1 ml distilled water. An aliquot of partially purified ICL virus was added to the stroma and the mixture allowed to stand at room temperature for several minutes. The mixture was centrifuged to precipitate the stroma and attached virus. The precipitate was resuspended and prepared for electron microscopy by the lowered drop method of Pinteric and Taylor (1962). The resulting electronphotomicrograph is shown in Fig. 4.



Log Haemagglutination Titre
3
2
1
5
10
20
30
40
50
60
5
10
20
30
40
Adsorption Time at 37°C
Elution Time at 4°C

Fig. 3

Adsorption and Elution of ICL Virus to Human 'O' RBC

(a) Adsorption: 1.5 ml of washed human 'O' RBC were added to 30 ml of virus suspension (HA titre 128) at 37° C in a water bath. At the adsorption times indicated, aliquots of the virus-RBC suspension were removed and centrifuged to precipitate the RBC and attached virus. The supernatant was assayed for HA.

(b) Elution: The virus-RBC precipitates from the adsorption experiments were diluted to the original volume with saline at 4° C and aliquots removed at the time shown, centrifuged, and the supernatant assayed for HA.

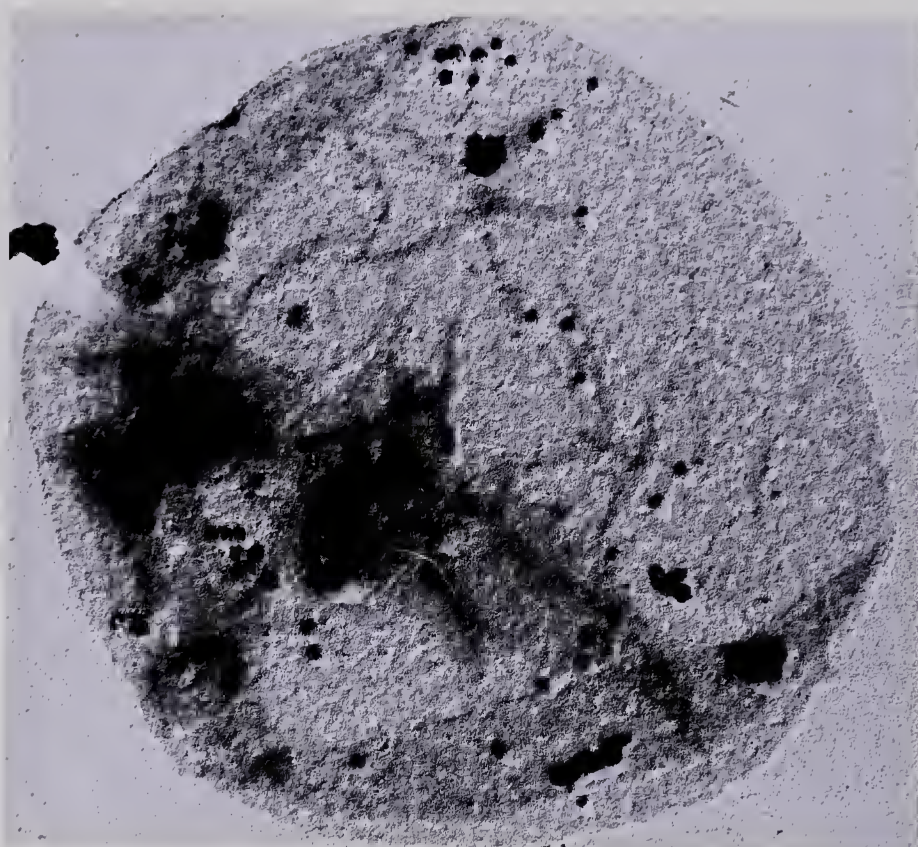


Fig. 4

Attachment of ICL Virus to RBC Stroma

A suspension of ICL virus was incubated with RBC stroma for several minutes at room temperature. The stroma and attached virus were sedimented and prepared for electron microscopy by the lowered drop method of Pinteric and Taylor (1962). The preparation was shadowed with palladium. The dark spheres are virus particles attached to the RBC stroma. x 20,000.

Effect of Sonication on ICL Virus Haemagglutinin

A stock suspension of ICL virus (HA titre 128) was used to determine the effect of sonic oscillation on the stability of the ICL virus HAn. Sonication was carried out as described under Materials and Methods, and the results obtained are shown in Table III.

TABLE III

Sonication Time in Seconds	HA Titre
0	128
30	512
60	32
90	4
120	4
180	0
240	0
300	0

An initial increase in HA was observed, but further sonication resulted in a marked decrease in HA. No HA was demonstrable after 180 seconds, indicating the marked lability of ICL virus to sonication.

Effect of CsCl on ICL Virus Haemagglutinin

As CsCl was initially used in the purification procedure of ICL virus, the effect of CsCl on ICL virus HAn was determined. A stock suspension of ICL virus (HA titre 2048) was used to determine the effect of CsCl on the stability of ICL virus HAn. The stock suspension was made 60 % with CsCl (the concentration of CsCl, buoyant density 1.40, normally used during the virus purification procedure) and allowed to stand at room temperature. The pH of the CsCl suspension was 6.8. The suspension was assayed for HA at 1, 3, 7, 14 and 21 days. No decrease in HA occurred indicating that CsCl had no deleterious effect on the virus.

Effect of Fluorocarbon on ICL Virus Haemagglutinin

Various workers have reported the use of fluorocarbon compounds in the purification procedure of adenoviruses (Green and Pina, 1963; Norrby, 1966; Valentine and Pereira, 1965). Attempts made in this study to utilize this method of purification resulted in loss of HA. The fluorocarbon used was "Freon TF" (trifluorotrichloroethane; Dupont of Canada, Ltd. Maitland, Ont.). A study of the effect of this fluorocarbon on a stock suspension of ICL virus (HA titre 65,536) was carried out (see Table IV) using the following procedures: an equal volume of fluorocarbon was added to the virus suspension in an Erlenmeyer flask and the flask shaken vigorously for the indicated time intervals, or, equal volumes of fluorocarbon and virus were mixed and blended in a Sorvall Omnimixer. In each case, the mixture was quickly centrifuged in a Sorvall RC2 centrifuge at 2,000 rpm for 10 minutes in order to separate the aqueous and organic layers. The aqueous layer, containing the virus, was removed and assayed for HA. Table IV shows results obtained by blending in the Omnimixer. After 30 seconds of blending, the HA titre is considerably reduced from that of the original suspension and is completely inactivated within 1 minute. Similar results were obtained by shaking the virus and fluorocarbon in a flask.

TABLE IV

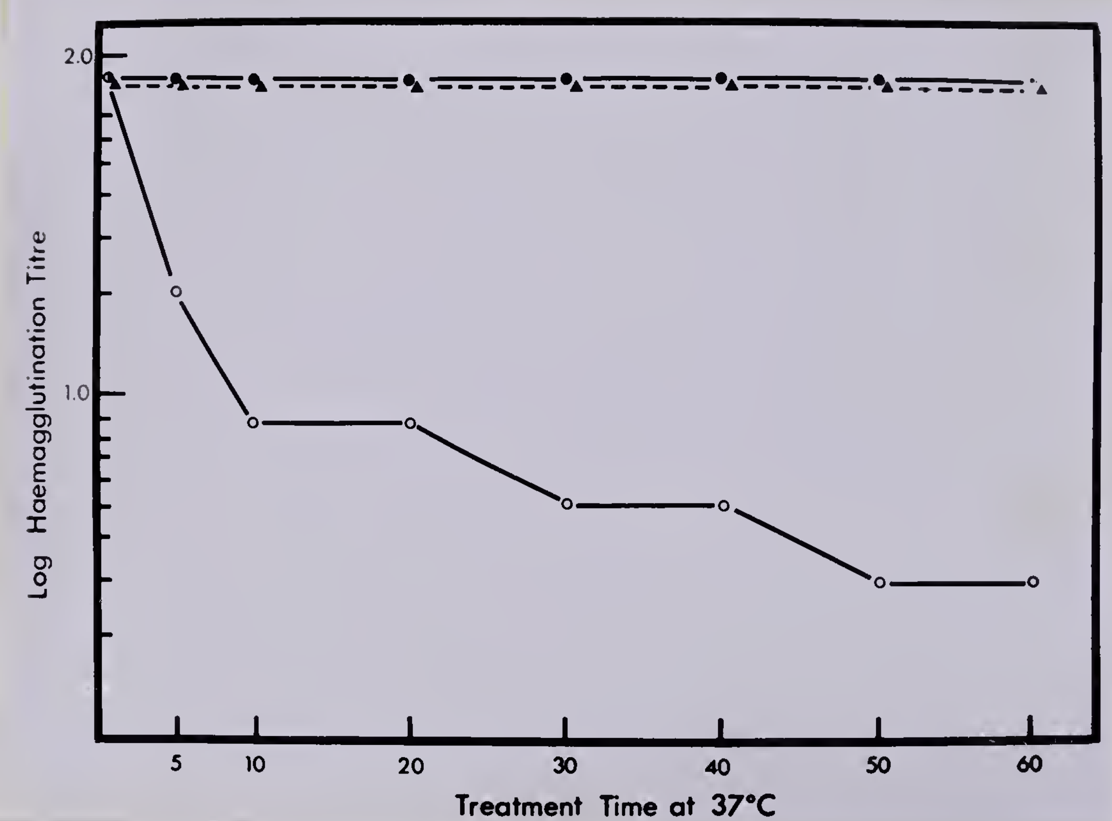
Fluorocarbon Treatment Time	HA Titre
0	65,536
30 seconds	512
60 seconds	0
120 seconds	0

Effect of Trypsin on RBC Receptors for ICL Virus

To obtain information on the nature of the red blood cell receptor taking part in the haemagglutination reaction, the red blood cells were treated with trypsin. The experiment was conducted according to the method of Simon (1962a). 25 ml of a 0.25 % trypsin solution (Matheson, Coleman and Bell, Norwood, Ohio) was added to 25 ml of a 1.0 % suspension of RBC and incubated at 37° C. 5 ml aliquots were removed at varying time intervals (0.5, 5, 10, 30 and 40 minutes) and 5 ml of cooled saline were added to stop the trypsin effect. The RBC were then made 0.5 % in saline and used for HA assays. Fig. 5 shows that trypsin elicited a decrease in the HA titre proportional to the exposure time. The titre of the HA system decreased from 64 to 2 in 60 minutes of treatment at 37° C.

Effect of Trypsin on ICL Virus Haemagglutinin

Fig. 5 shows that ICL virus exposed to trypsin under the same conditions as the RBC did not change in its capacity to agglutinate untreated RBC. Aliquots of an ICL virus suspension were incubated with 0.25 % trypsin for 1 hour at 37° C. Samples removed at varying time intervals were diluted with equal volumes of cooled saline and used for HA assay.



2.0
1.0
Log Haemagglutination Titre
5
10
20
30
40
50
60
Treatment Time at 37°C

Fig. 5

<u>Effect of Typsin on RBC Receptor and ICL Virus Haemagglutinin</u>

25 ml of a 0.25 % trypsin solution was added to 25 ml of a 1.0 % suspension of RBC and incubated at 37° C. 5 ml aliquots were removed at the indicated times and 5 ml of cold saline added to stop the trypsin effect. The RBC were made 0.5 % in saline and used for HA assay. Aliquots of ICL virus were treated in the same manner.

Closed circles: Trypsin treated ICL virus
Open circles: Trypsin treated RBC
Triangles: Untreated ICL virus and RBC

pH Stability of ICL Virus Haemagglutinin at Room Temperature

A crude virus suspension (8,000 rpm supernatant from the purification procedure) was centrifuged at 20,000 rpm for 2 hours in a Spinco #30 rotor. The pellet was resuspended in ion-exchange water and diluted to an HA titre of 64.

Aliquots of ion-exchange water were adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with dilute HCl or NaOH solutions. 5 ml aliquots of the virus suspension were added to 5 ml of the pH adjusted ion-exchange water. 0.25 ml aliquots were removed at the time intervals indicated in Table V and assayed for HA.

The final pH of each of the virus suspensions was measured and is shown in Table V.

HA assays were done in the standard manner using phosphate buffered saline (PBS) at pH 7.2.

TABLE V

pH	Exposure Time in Minutes at Room Temperature						
	5	10	20	30	60	120	240
2.3	32	32	16	16	16	16	8
2.9	32	32	32	16	16	16	8
4.2	32	32	32	16	16	16	8
5.2	32	32	32	16	16	16	8
6.3	32	32	32	32	32	32	32
7.1	32	32	32	32	32	32	32
8.1	32	32	32	32	32	32	32
9.2	32	32	32	32	32	16	32
9.8	32	32	32	32	16	32	16

It is apparent that the HA of ICL virus is relatively stable over a wide pH range at room temperature. Surprisingly, low and high pH values of 2.3 and 9.8 did not completely inactivate the HA activity of the virus. It would appear from the results obtained for the 240 minute period that the optimum pH range is 6 - 9.

Incubation at these pH values for as long as 24 hours give little change in HA titre.

Purification and Concentration of ICL Virus

Numerous procedures have been reported concerning the purification of adenoviruses. However, of the several purification methods attempted in this study (fluorocarbon extraction, methanol precipitation, column chromatography, density gradient equilibrium centrifugation), most did not give yields of ICL virus of sufficiently high HA titre to warrant their use in the purification of the virus so that its agglutinating property could be studied.

As stated previously, the commonly used method of fluorocarbon extraction resulted in loss of all detectable activity (see Table IV). Further, the method of choice which had been used previously to this study (Gaunt, 1966), was to purify the ICL virus by differential centrifugation of virus tissue culture harvest. This method yielded very low HA titres. Therefore, it was necessary to design a procedure whereby large amounts of ICL virus could be attained which would still retain a high HA activity.

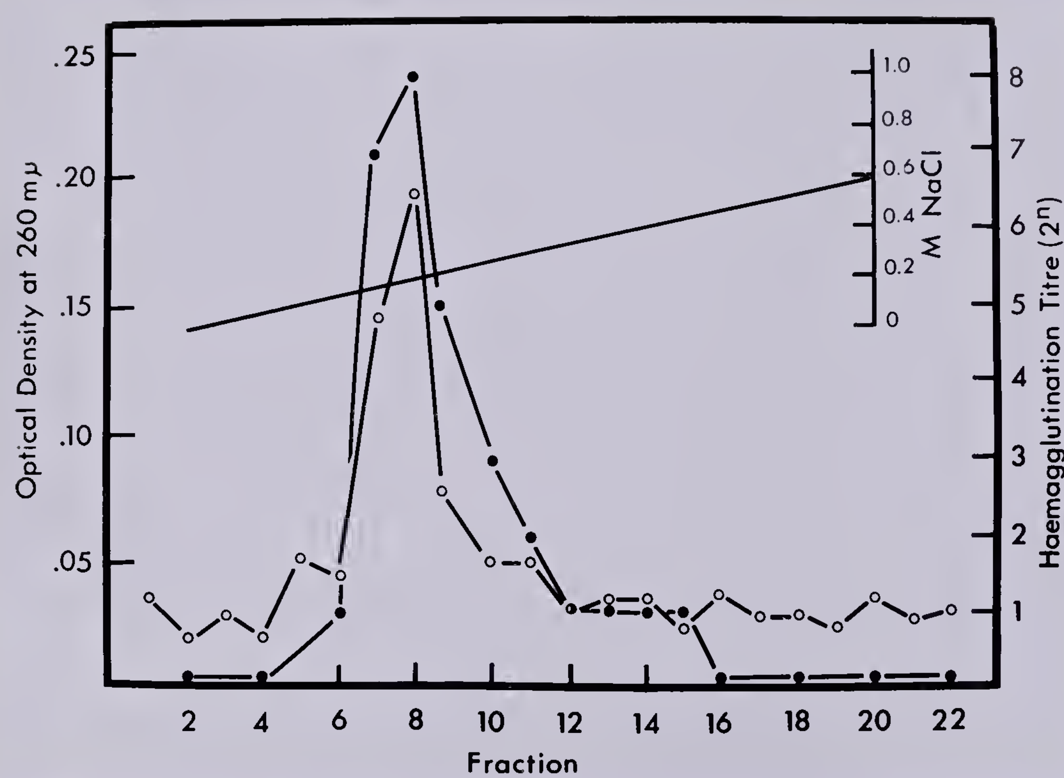
Several methods of column chromatography were attempted in order to purify the ICL virus present in a crude virus suspension. A DEAE-cellulose column (15 x 1.3 cm) was prepared as outlined in Materials and Methods and a crude virus suspension (10 ml; HA titre 1024) was added and

the column washed with several volumes of buffer (0.01 M Tris-HCl pH 7.2). The column was then eluted with a linear gradient of NaCl in 0.01 M Tris-HCl pH 7.2). The result of this purification attempt is seen in Fig. 6. A single optical density peak at 0.2 M NaCl was obtained which also contained the haemagglutinin. Electron microscopic examination of the fractions with the highest optical density at 260 mμ and exhibiting the highest HA activity, revealed that the eluent (at 0.2 M NaCl) was not sufficiently purified or concentrated to warrant further study.

Calcium phosphate - cellulose chromatography was carried out in the manner of Simon (1962b) but did not prove successful as the virus was not adsorbed.

Purification and concentration in sucrose density gradients was also attempted. Linear gradients of 5 - 20 % sucrose in 0.01 M Tris HCl buffer pH 7.2 were prepared using a Buchler density gradient apparatus (Buchler Instruments, Fort Lee, N.J.). The crude virus suspension was layered on top of the prepared gradient and centrifuged at 10,000 rpm for 15 minutes in a Spinco SW25.2 rotor. After centrifugation, a broad band of virus was obtained in the middle of the centrifuge tube. The band was quite disperse and collection of fractions by the drop method resulted in a very wide spread distribution of virus material. Consequently, adequate concentration of the virus material was not achieved.

The methanol precipitation method of Wilox and Ginsberg (1963) was attempted but this too resulted in a loss of HA activity.



.25
.20
.15
.10
.05
Optical Density at 260 mμ
1.0
0.8
0.6
0.4
0.2
0
M NaCl
8
7
6
5
4
3
2
1
Haemagglutination Titre (2ⁿ)
2
4
6
8
10
12
14
16
18
20
22
Fraction

Fig. 6

Purification of ICL Virus with DEAE-cellulose

10 ml of a crude virus suspension (HA titre 1024) were added to a DEAE-cellulose column (1.3 x 15 cm) and eluted with a linear gradient of NaCl in 0.01 M Tris-HCl, pH 7.2. Electron microscopic examination of the 0.2 M NaCl eluent revealed that both virus and cell debris were present.

Closed circles: HA titre
Open circles: Optical density

CsCl density gradient centrifugation (Meselson et al, 1957) was the method of choice in purifying ICL virus. Previous studies carried out in this laboratory with ICL virus had shown that a large recovery of virus could be achieved using this method. Gaunt (1966) when using this method had managed to purify sufficiently large numbers of virus to carry out chemical analysis, but the HA was largely inactivated. Prior to the final CsCl density gradient centrifugation however, the virus had been centrifuged to a pellet and resuspended in buffer. It was thought that this centrifuging to a pellet might have had some bearing on the decrease in HA titre. Norrby et al (1964) have reported a method for the purification of human adenovirus 3 which utilizes a CsCl cushion during the centrifugation studies. This method was adopted in order to see if higher yields of the haemagglutinating activity could be obtained.

Crude virus suspensions were prepared as described in Materials and Methods and re-centrifuged at 19,000 rpm for 6 hours to precipitate the ICL virus. It was found that shorter time intervals did not completely precipitate all the HA activity. The pellet from the 6 hour centrifugation was resuspended in 60 ml of buffer and sonicated for 30 seconds to disrupt the clumps. The suspension was then diluted to 150 ml with buffer and 50 ml aliquots were layered on a 10 ml CsCl solution (60 %). The tubes were centrifuged at 20,000 rpm for 1 hour and a band of partially purified virus was obtained (Fig. 7). This band was removed using a 4-inch canula fitted to a 2 ml disposable syringe. In some instances it was not possible to remove this band without contamination with some of the adjacent protein band. The virus was dialyzed to remove the CsCl. The

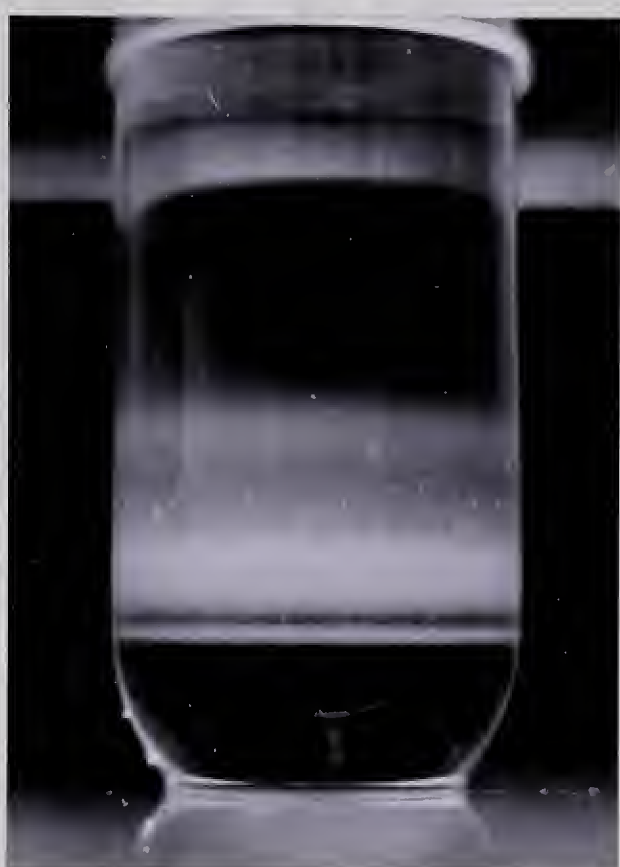


Fig. 7

Separation of ICL Virus by Sedimentation onto a CsCl Layer

50 ml of the 19,000 rpm pellet resuspension were layered on 10 ml of 60 % CsCl and centrifuged at 20,000 rpm for 1 hour in a Spinco SW25.2 rotor. The band nearest the bottom of the tube consists of ICL virus.

material was then submitted to a final CsCl density gradient centrifugation.

Three methods of preparing CsCl density gradients were utilized. First, for CsCl density gradient equilibrium centrifugation, 5 ml of the virus suspensions which were to be purified were made approximately 55 % with CsCl (in a Spinco SW39 centrifuge tube) or until a buoyant density of 1.34 was achieved. The tubes were centrifuged at 35,000 rpm (Spinco Model L2 centrifuge, Beckman Inst., Palo Alto, California) in a Spinco SW39 rotor for 48 hours at 6° C.

Second, a linear gradient of 4 ml CsCl from 1.30 to 1.38 buoyant density was prepared with the Buchler density gradient apparatus and the virus suspension under study (1.5 ml) was layered on top of the gradient. The prepared gradients were then centrifuged in a Spinco SW39 rotor at 35,000 rpm for 3 hours at 6° C.

Third, a non-linear gradient of CsCl was prepared by successively layering 1 ml volumes of CsCl solutions of 1.40, 1.36, 1.32, and 1.29 g/ml buoyant density in a SW39 rotor centrifuge tube and 1.5 ml of the virus suspension were layered on top of the gradient. The gradients were centrifuged for 3 hours at 35,000 rpm at 6° C. All three methods of preparing CsCl density gradients gave similar results. Fig. 8 illustrates the virus bands obtained after centrifugation. Fractions were collected by puncturing the centrifuge tube with a Buchler drop collecting unit (Buchler Instruments, Fort Lee, N.J.). Following buoyant density determination, the fractions were diluted to the desired volume with buffer and optical density at 260 mμ, HA and infectivity determined.

The bands were sufficiently far apart to separate the virus from the bulk of the cellular protein, but in some instances there was a

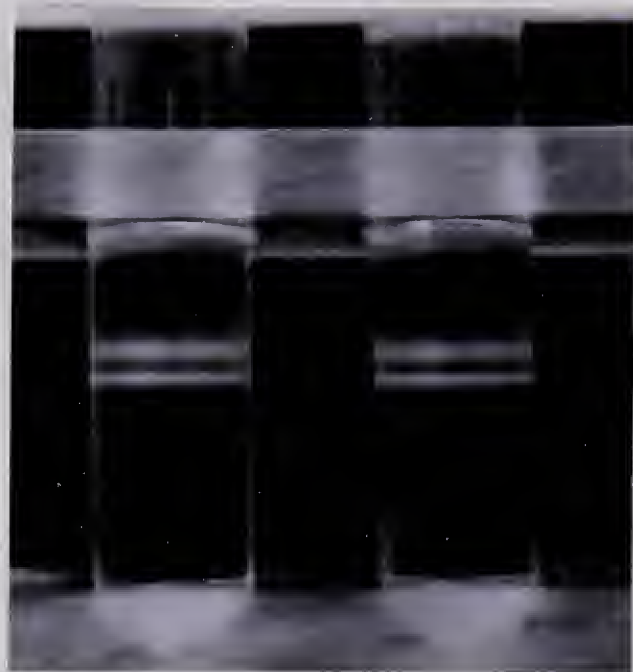


Fig. 8

Purification of ICL Virus by Sedimentation in CsCl Density Gradients

Upper: Purification of ICL virus by centrifugation to equilibrium in a CsCl solution of initial buoyant density 1.34 g/ml. The bottom band contains ICL virus. 35,000 rpm for 48 hours at 6° C in a Spinco SW39 rotor.

Middle: Purification of ICL virus by centrifugation in a CsCl linear gradient of 1.30-1.38 g/ml. 35,000 rpm for 3 hours

Lower: Purification of ICL virus by centrifugation in a preformed CsCl non-linear gradient, of 1.40, 1.36, 1.32, 1.29 g/ml 35,000 rpm for 3 hours.

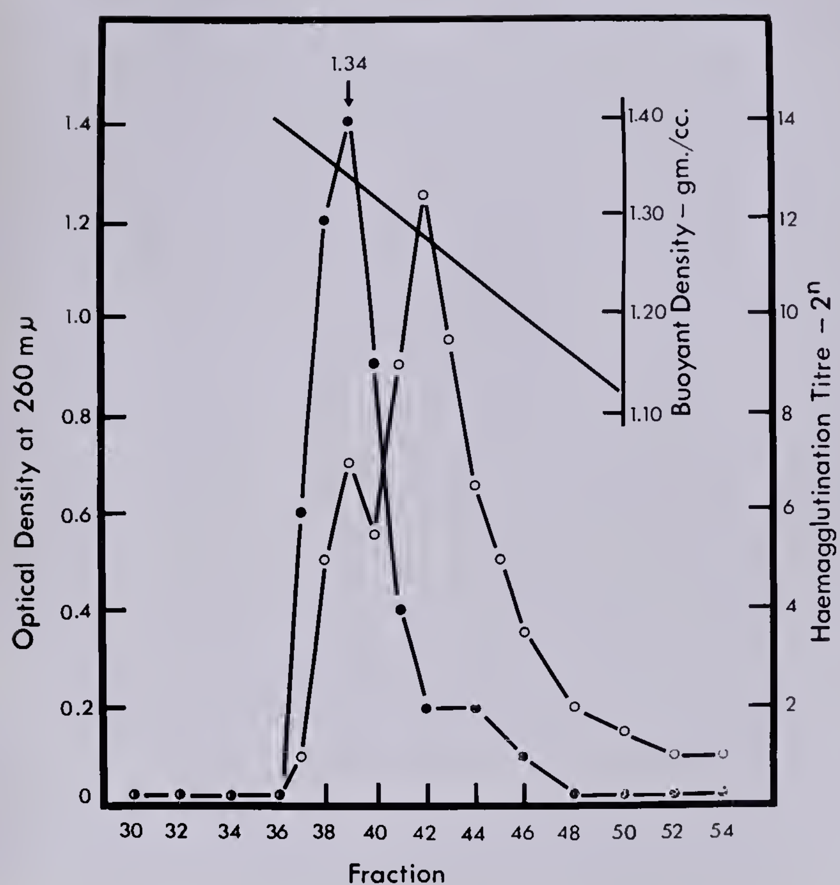
certain amount of overlap in the two bands. When separation was incomplete, as shown in Figs. 9 and 10, the virus fractions were pooled, concentrated, and subjected to a second CsCl density gradient centrifugation. Figs. 11, 12 and 13 show the results obtained with linear gradients and with equilibrium density gradient centrifugation. It is seen that the virus obtained in this manner is essentially free from cellular protein, is infectious, and has a high HA titre.

Fig. 14 shows the results obtained when a partially degraded virus preparation (spontaneous degradation occurring during storage at 4° C) was subjected to CsCl density gradient centrifugation. Those fractions showing HA and elevated optical density values were assayed for infectivity. Three optical density peaks were obtained, but only the first peak was associated with infectivity and HA.

Electron microscopic examination of the peaks shown in Fig. 14 revealed that peak 1 was composed of intact virus particles, peak 2 of capsomere aggregates with a few intact virus particles, and peak 3 of individual capsomeres. Although buoyant density determinations were not done, other experiments have shown that the virus has a buoyant density of 1.34 g/ml. The other two components, therefore, would have a buoyant density less than 1.34 g/ml.

A scheme of the purification method of ICL virus which was developed in this study is shown in Fig. 15. Prior to the 8,000 rpm 15 minute centrifugation, the infected tissue culture was subjected to three cycles of freeze thawing. The final suspension of purified virus was made 0.01 % with bovine serum albumin (Pentex Inc., Kankakee, Ill.) in order to minimize viral aggregation and degradation (Norrby, 1966).

Table VI shows the per cent recovery of HA which accompanies the virus purification and concentration procedure.


1.34
Optical Density at 260 mμ
Buoyant Density – gm./cc.
Haemagglutination Titre – 2ⁿ
Fraction

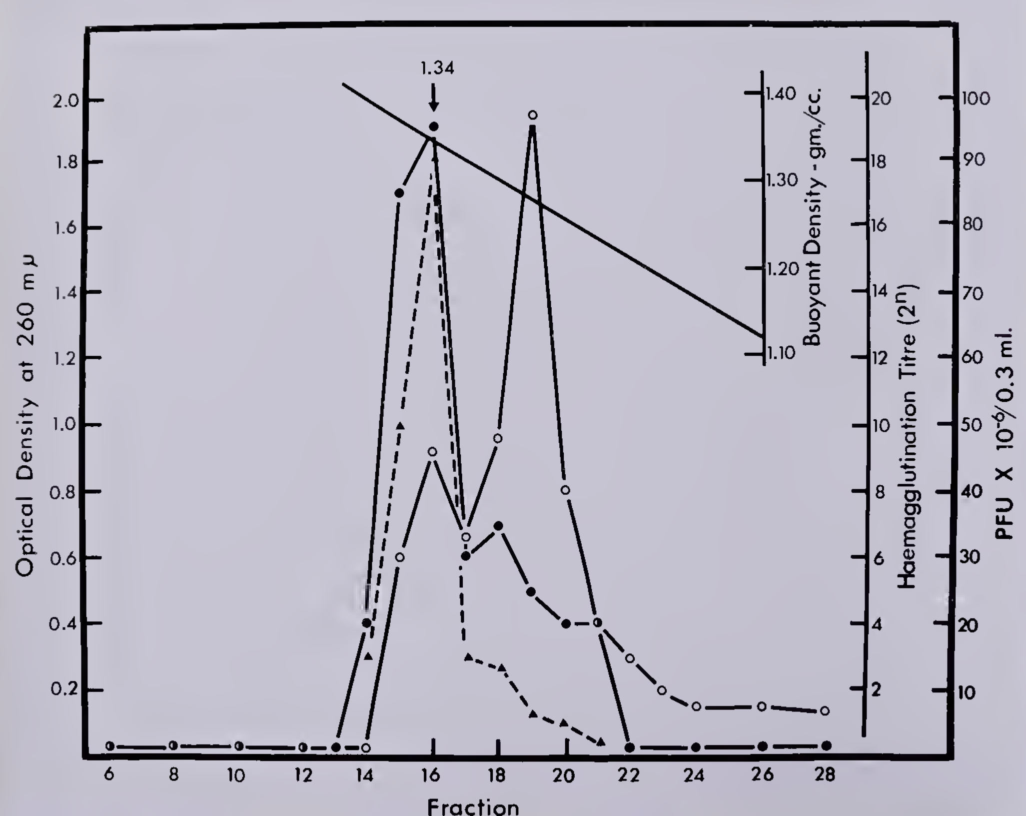

1.34
Optical Density at 260 mμ
Buoyant Density - gm./cc.
Haemagglutination Titre (2ⁿ)
PFU X 10⁻⁶/0.3 ml.
Fraction

Fig. 9

Partial Separation of ICL Virus from Cellular Protein

A dialyzed virus-protein suspension from the CsCl cushion purification step (Fig. 7), was centrifuged in a preformed CsCl linear density gradient at 35,000 rpm for 3 hours. There was an overlap in the peaks containing virus and protein.

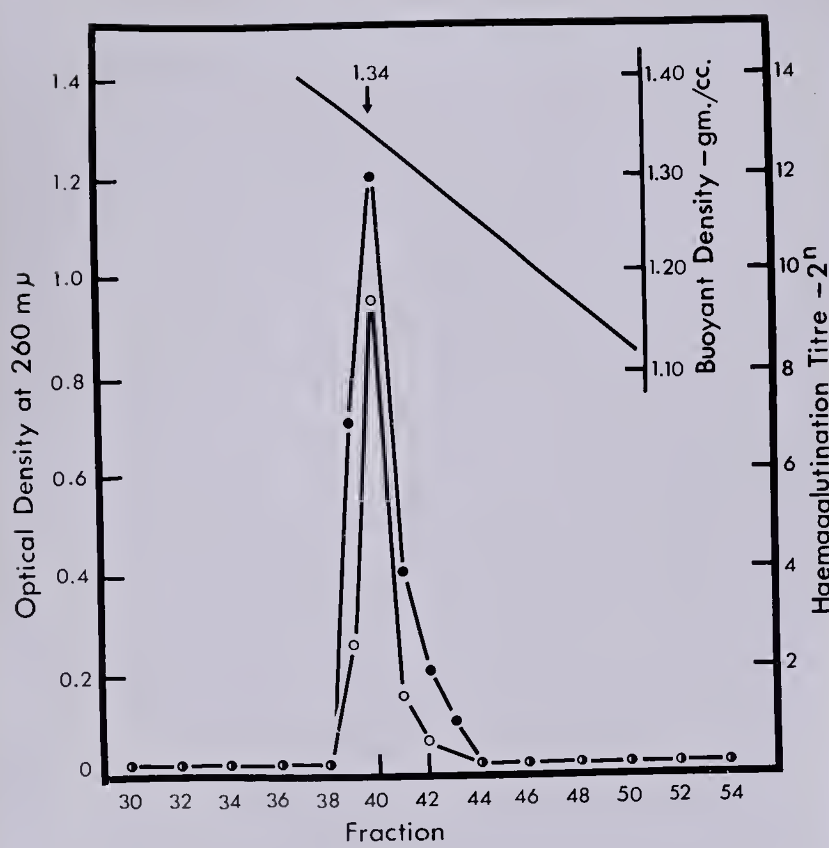
Closed circles: HA titre
Open circles: optical density

Fig. 10

Partial Separation of ICL Virus from Cellular Protein

Same conditions as Fig. 9, except an infectivity assay was also carried out.

Closed circles: HA titre
Open circles: optical density
Triangles: infectivity


1.34
Optical Density at 260 mμ
Buoyant Density – gm./cc.
Haemagglutination Titre – 2^n
Fraction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1.40
1.30
1.20
1.10
14
12
10
8
6
4
2
30 32 34 36 38 40 42 44 46 48 50 52 54

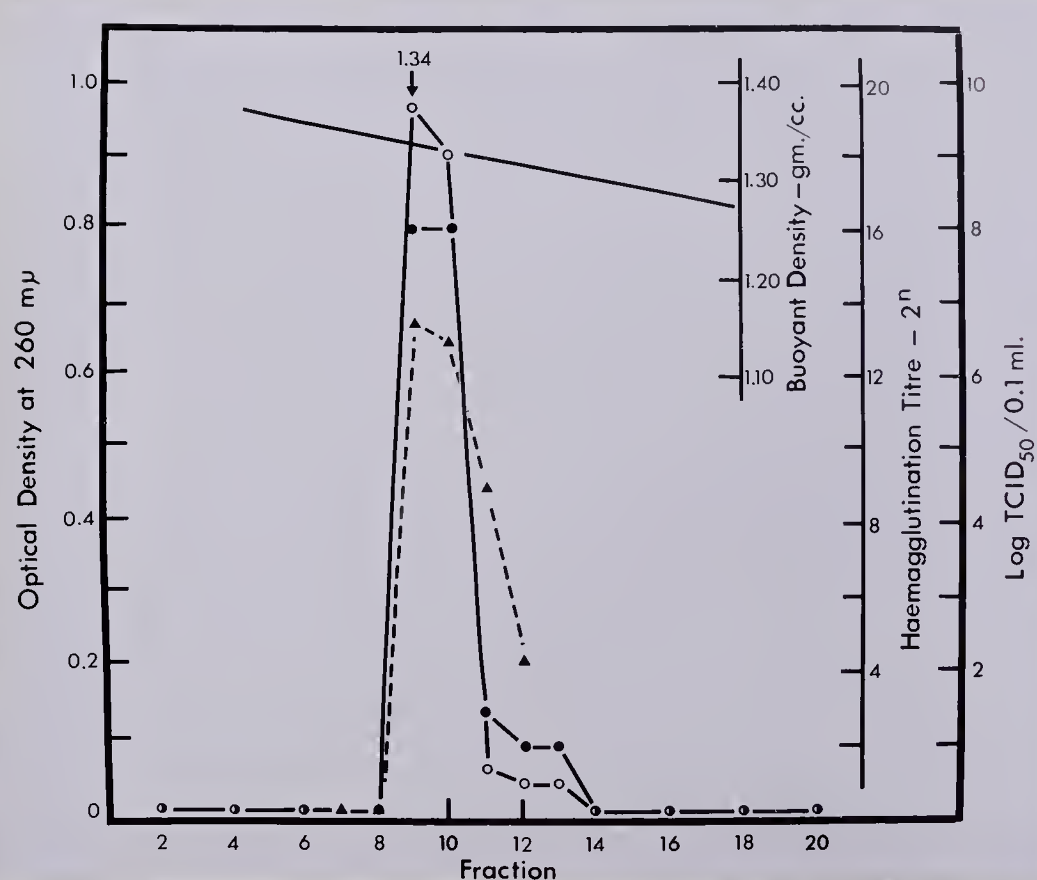

1.34
Optical Density at 260 mμ
Buoyant Density – gm./cc.
Haemagglutination Titre – 2^n
Log $TCID_{50}$ /0.1 ml.
Fraction
1.0
0.8
0.6
0.4
0.2
0
1.40
1.30
1.20
1.10
20
16
12
8
4
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20

Fig. 11

CsCl Density Gradient Centrifugation of ICL Virus

Material obtained from fractions showing viral activity in Fig. 9 and 10 was further centrifuged on a preformed CsCl linear density gradient of buoyant density 1.30 - 1.38 g/ml. 35,000 rpm for 3 hours.

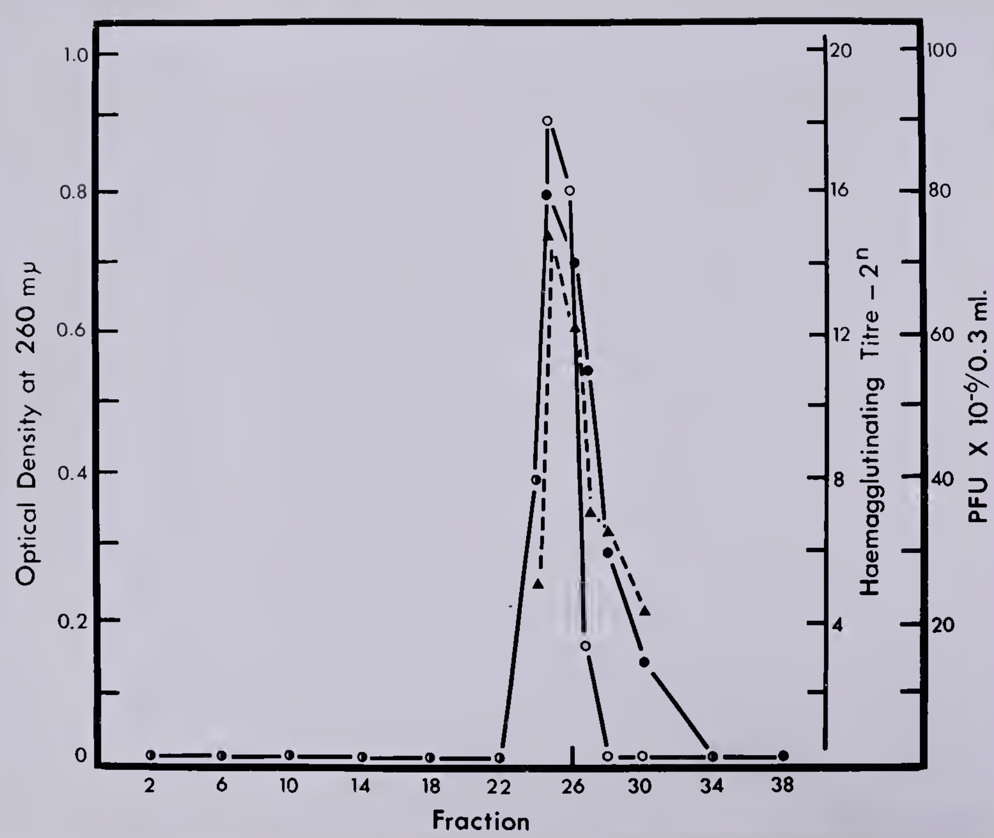
Closed Circles: HA titre
Open Circles: optical density

Fig. 12

CsCl Density Gradient Centrifugation of ICL Virus

Same conditions as in Fig. 11, except that the virus material was centrifuged to equilibrium in a CsCl solution of initial buoyant density 1.34 g/ml. 35,000 rpm for 48 hours.

Closed Circles: HA titre
Open Circles: optical density
Triangles: infectivity



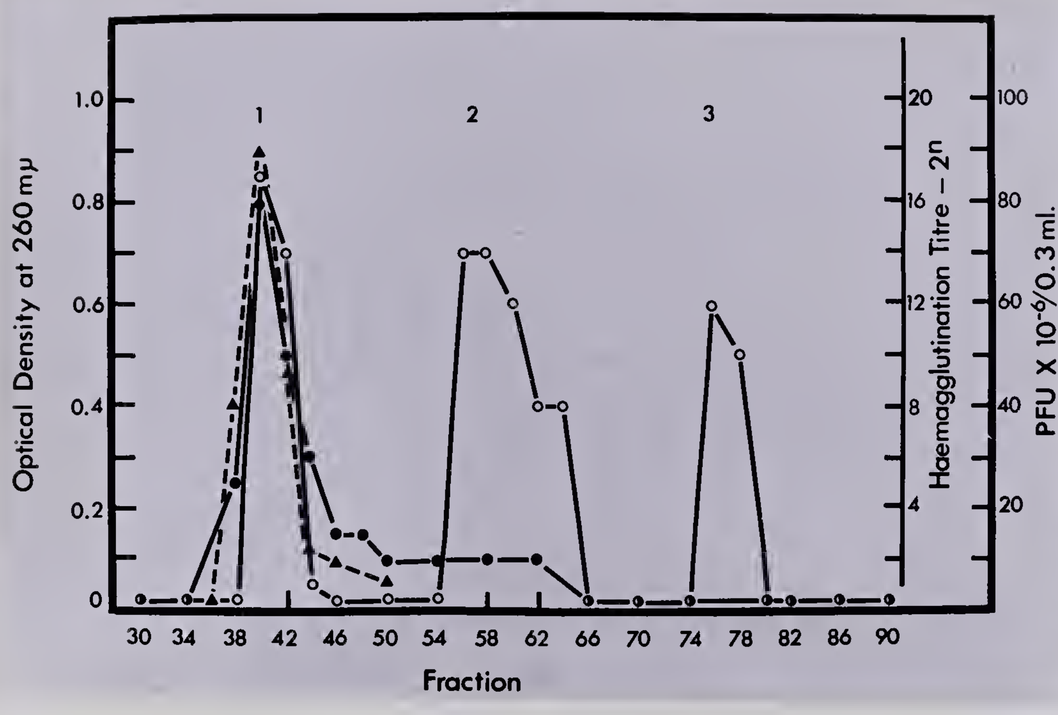
Optical Density at 260 mμ
1.0
0.8
0.6
0.4
0.2
0
Haemagglutinating Titre – 2^n
20
16
12
8
4
PFU X 10^{-6}/0.3 ml.
100
80
60
40
20
2
6
10
14
18
22
26
30
34
38
Fraction

Fig. 13

<u>CsCl Density Gradient Centrifugation of ICL Virus</u>

Same conditions as in Fig. 11, except that the virus material was layered on a preformed CsCl non-linear density gradient of buoyant density 1.40 - 1.36 - 1.32 - 1.29 g/ml.

Closed Circles: HA titre
Open Circles: optical density
Triangles: infectivity



Optical Density at 260 mμ
1.0
0.8
0.6
0.4
0.2
0
1
2
3
Haemagglutination Titre – 2n
20
16
12
8
4
PFU X 10-6/0.3 ml.
100
80
60
40
20
30 34 38 42 46 50 54 58 62 66 70 74 78 82 86 90
Fraction

Fig. 14

Purification of Partially Degraded ICL Virus by CsCl Density Gradient Centrifugation

Partially degraded ICL virus was subjected to CsCl density gradient centrifugation at 35,000 rpm for 3 hours. Peak 1 consisted of complete virus particles, peak 2 of capsomere aggregates, and peak 3 of individual capsomeres. Infectivity and haemagglutination are associated only with the intact virus.

Closed Circles: HA titre
Open Circles: optical density
Triangles: infectivity

Infected Tissue Culture Harvest

↓ 8000 RPM 15 min.

Supernatant

↓ 19000 RPM/6 hr.

Pellet

↓ Resuspend

Suspension

CsCl Cushion ↓ 20000 RPM/1 hr.

'Band'

Dialysis ↓ Concentration

Concentrate

CsCl Density Gradient ↓ 35000 RPM/3 hr.

'Band'

Dialysis ↓

Store at 4°C
in 0.01% BSA

Fig. 15

Schematic Representation of ICL Virus Purification Procedure

Prior to the 8,000 rpm 15 minute centrifugation, the infected tissue culture harvest was subjected to three cycles of freeze-thawing at -70° C and room temperature.

TABLE VI

Purification Step	HAU/volume
Infected TC harvest	1.54×10^6/1.5 liters
8,000 rpm spnt	1.54×10^6/1.5 liters
19,000 rpm pellet	2.44×10^6/150 ml
CsCl cushion band	5.24×10^6/4 ml
CsCl density gradient band	2.82×10^5/4 ml

A recovery of 18.3 % of the total HA in the original infected tissue culture harvest was obtained. Even though the HA had been sufficiently concentrated to give high titres, the overall recovery was not excessive.

Calculation of per cent recovery of infectivity of ICL virus in the purification procedure was calculated from the results shown in Fig. 12. The per cent recovery of infectivity was calculated as follows:

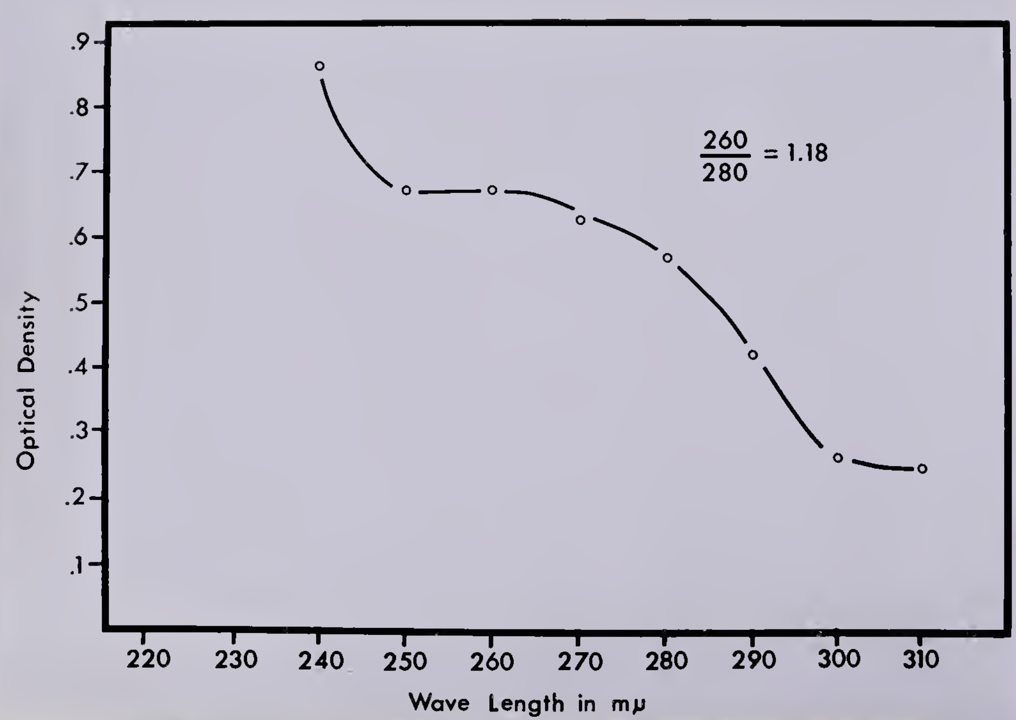
Sample Assayed	Infectivity	
8,000 rpm spnt (1.5 liters)	6.37×10^5/ml	= 9.56×10^8 $TCID_{50}$/1.5 liters
CsCl Fraction 9 (10 ml)	6.8×10^6 $TCID_{50}$/ml	= 68.0×10^6/10 ml
10 (10 ml)	6.5 x " $TCID_{50}$	65.0 "
11 (10 ml)	4.5 x " $TCID_{50}$	45.0 "
12 (10 ml)	2.1 x " $TCID_{50}$	21.0 "
		199.0×10^6 $TCID_{50}$ recovery

Per cent recovery of infectivity after CsCl density gradient centrifugation:

$$\frac{199.0 \times 10^6 \ TCID_{50}}{9.56 \times 10^8 \ TCID_{50}} \times 100 = 20.8 \%$$

UV Absorption Spectrum of Purified ICL Virus

One ml of purified ICL virus (Fig. 16) was used to determine the absorbance of purified ICL virus between wave lengths 200 mμ and 320 mμ. The 260:280 ratio of the purified virus was found to be 1.18. There was no definite peak at 260 mμ but a definite shoulder was obtained.



.9
.8
.7
.6
.5
.4
.3
.2
.1
Optical Density
$\frac{260}{280} = 1.18$
220
230
240
250
260
270
280
290
300
310
Wave Length in mμ

Fig. 16

UV Absorption Spectrum of Purified ICL Virus

Plaque Assay of ICL Virus

Prior to the beginning of this study, a plaque assay system had been established whereby ICL virus infectivity could be assayed on monolayers of DKL cells (Gaunt, 1966). However, the assay system ceased to be operative. It was thought that the incubation period for plaque formation was longer than the viable life span of the cell monolayer under the conditions of the assay system. Upon receipt of another cell line, MDC, further attempts were made to re-establish a workable plaque assay system.

Preliminary infectivity assays using the procedure reported by Gaunt (1966) failed to produce plaques. Certain modifications in handling of the infected cell monolayers were adopted. MDC cell monolayers were prepared in disposable tissue culture plates as outlined in Materials and Methods. When the monolayer was complete, the medium was removed and the monolayer washed with physiological saline. 0.3 ml of the virus suspension to be assayed was added to the monolayer and adsorption allowed to take place for 60 minutes at 37° C. The plates were rotated gently at 15 minute intervals. After adsorption, the cell monolayer was covered with a melted agar-medium suspension prepared in the following manner: 50 ml of 3 % Noble agar in ion-exchange water were melted and cooled to 43° C in a water bath. 2X MEM containing 20 % calf serum, twice the normal concentration of antibiotics, and 3 ml of 7.5 % sodium bicarbonate solution (to adjust the pH) was heated to 40° C. The two solutions were mixed by pouring the heated medium into the cooled agar. The agar-medium mixture was then added to the cell monolayers in a quantity just sufficient to cover the cells. After the overlay had hardened, the plates were inverted and incubated at 37° C

in an atmosphere of 4 % CO_2 and 96 % air. On the fourth day after infection, a similar overlay was added and on the seventh day a third overlay containing a 1:10,000 dilution of neutral red stain and no calf serum was added. The plates were incubated for two more days and examined for produced plaques.

The pH of the original agar-medium overlay was checked during the incubation period and was found to become acidic about the fourth day after infection. In order to alleviate this drop in pH, which resulted in loss of cell viability, it was necessary to introduce a second agar-medium overlay into the procedure on the fourth day after infection.

The effect of HLA in the overlay medium was investigated. An overlay medium containing 50:50 MEM-HLA and 10 % calf serum was used. A second overlay was added on the fourth day after infection and a third, containing the stain, was added on the seventh day. Subsequent examination of the plates revealed plaque formation with a marked increase in plaque number. Fig. 17 shows the appearance of the plaques. Table VII shows a comparison of the plaque number obtained with and without HLA in the overlay medium.

TABLE VII

Virus Dilution	Plaque Number	
	MEM	MEM-HLA
10^{-6}	TNTC*	TNTC
10^{-7}	39	133
10^{-8}	7	14
10^{-9}	0	1

It appears that the addition of HLA in the overlay medium had a beneficial effect by increasing the number of visible plaques.

* Too numerous to count.

Fig. 17

<u>Plaque Assay of ICL Virus on MDC Cell Monolayer</u>

ICL infected MDC cells overlain with 50:50 MEM: HLA containing 1:10,000 neutral red as a vital stain.

Effect of Additives in the Plaque Assay

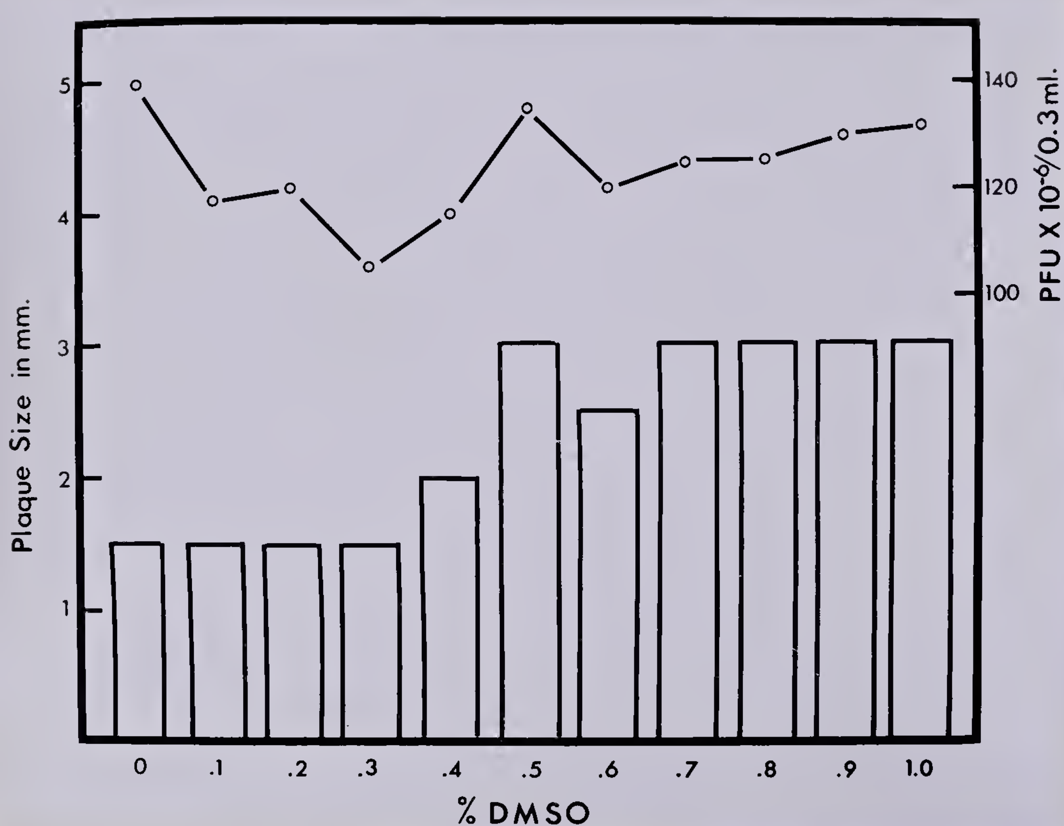
The effect of various additives to the overlay medium was studied. Additives chosen for study were those used in plaque assay by several workers (Colter et al, 1964; Liebhaber and Takamoto, 1963; Tovell and Colter, 1967). The additives used were protamine sulfate (Eli Lilly Co., Indianapolis, Indiana), Diethylaminoethyl-dextran (DEAE-dextran; Pharmacia, Uppsala, Sweden), and Dimethylsulfoxide (DMSO; Eastman Organic Chemicals, Rochester, New York). Previous studies (Gaunt, 1966) showed that the addition of protamine sulfate or DEAE-dextran to the overlay medium of the ICL-DKL cell plaque assay system would increase the plaque size but not the plaque number.

Experiments carried out with a wide range of DMSO concentration in the overlay medium described above, showed that plaque size was increased but plaque number was not markedly affected. Table VIII shows that in one experiment, 2 % DMSO in the overlay medium did increase the plaque number slightly whereas 5 % DMSO proved toxic to the cells. The slight increase in plaque number with 2 % DMSO may have been due to larger, more visible plaques.

TABLE VIII

Virus Dilution	Plaque Number		
	Control	2 % DMSO	5 % DMSO
10^{-6}	TNTC	TNTC	Toxic
10^{-7}	121	153	Toxic
10^{-8}	6	8	Toxic
10^{-9}	0	2	Toxic

To determine more accurately the effect of DMSO, a series of assays with the overlay medium containing 0.1 - 1.0 % DMSO were performed. The results are shown in Fig. 18. The plaque number remained

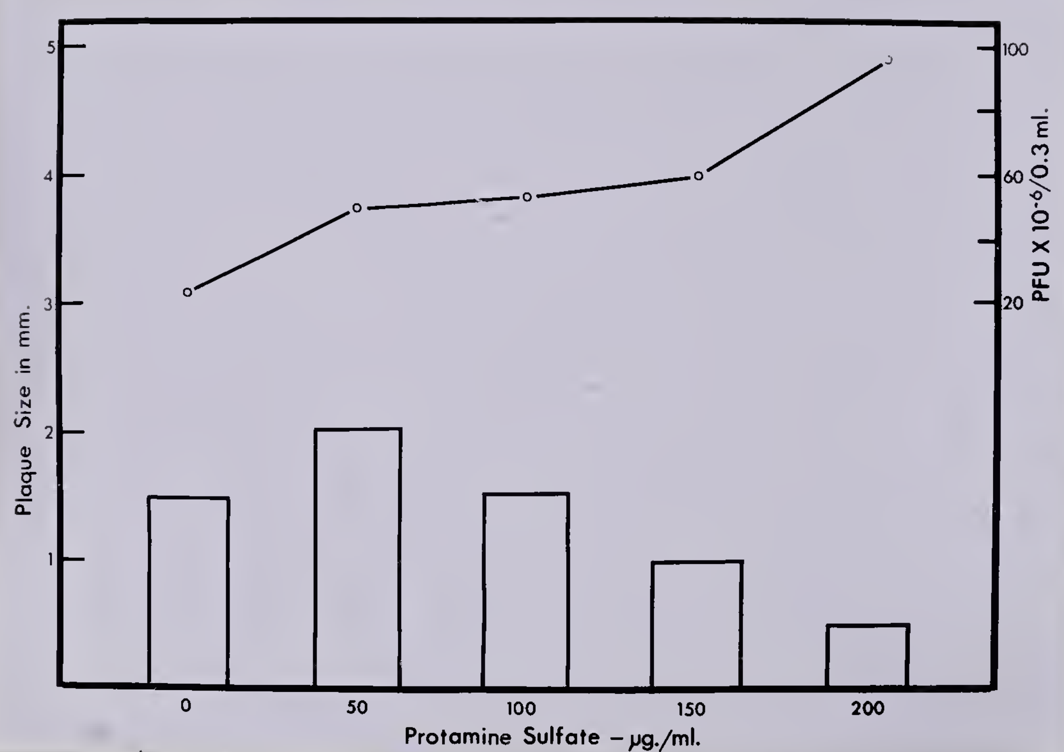


Plaque Size in mm.
5
4
3
2
1
140
120
100
PFU X 10^-6/0.3 ml.
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
% DMSO

Fig. 18

Effect of DMSO in Plaque Assay Overlay Medium

DMSO in concentrations ranging from 0.1 % to 1.0 % was added to the 50:50 MEM:HLA overlay medium of ICL virus infected MDC cells.

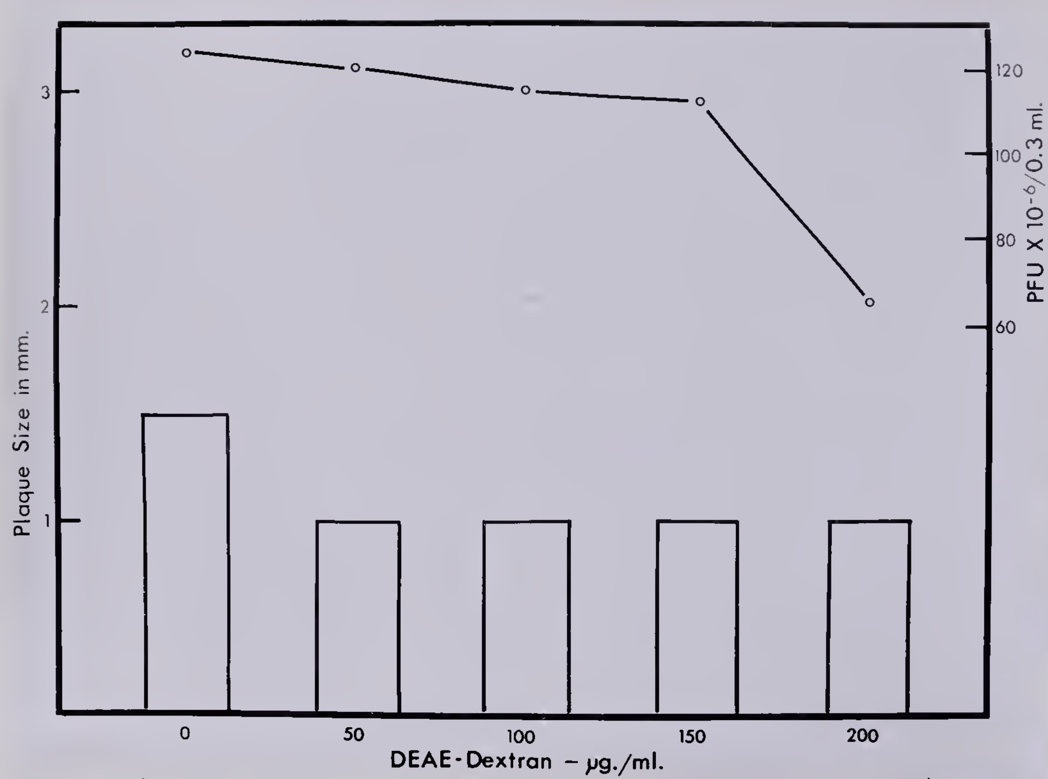


Plaque Size in mm.
5
4
3
2
1
100
60
20
PFU X 10⁻⁶/0.3 ml.
0
50
100
150
200
Protamine Sulfate – µg./ml.

Fig. 19

Effect of Protamine Sulfate in Plaque Assay Overlay Medium

Protamine sulfate in concentrations ranging from 50 - 200 μg/ml was added to the overlay medium of ICL virus infected MDC cells.



Plaque Size in mm.
3
2
1
120
100
80
60
PFU X 10⁻⁶/0.3 ml.
0
50
100
150
200
DEAE-Dextran – µg./ml.

Fig. 20

Effect of DEAE-Dextran in Plaque Assay Overlay Medium

DEAE-dextran in concentrations ranging from 50 - 200 μg/ml was added to the overlay medium of ICL virus infected MDC cells.

fairly constant over the concentration range but a noticeable increase in plaque size occurred at a DMSO concentration of 0.5 % or greater.

The effects of protamine sulfate and DEAE-dextran on the plaque assay were also investigated and are shown in Figs. 19 and 20. As the concentration of protamine sulfate in the overlay medium was increased, the plaque number increased but the plaque size diminished to less than 0.5 mm in diameter. The plaque number in the DEAE-dextran studies was not markedly affected up to a concentration of 150 μg/ml but decreased significantly at 200 μg/ml. The plaque size remained relatively constant over the range of DEAE-dextran used.

Relationship of Particle Count, HA Titre, and Infectivity with Purified Virus

Particle counts, HA assays and infectivity assays were carried out on purified ICL preparations. The particle counts were done as described in Materials and Methods (see also Fig. 21). Infectivity was determined as described in Materials and Methods. As the standard method of assaying for HA titre was not considered accurate enough to give precise values for these calculations, the corrected HA titre was obtained from the HA assay correction curve described in Materials and Methods and shown in Fig. 1. The number of virus particles per ml in three different purified ICL virus preparations was determined by using the calculated value of 3.46×10^{10} latex particles per ml. The values obtained are shown in Table IX. From these values, the ratios of virus particles per RBC were calculated. These values are also shown in Table IX.

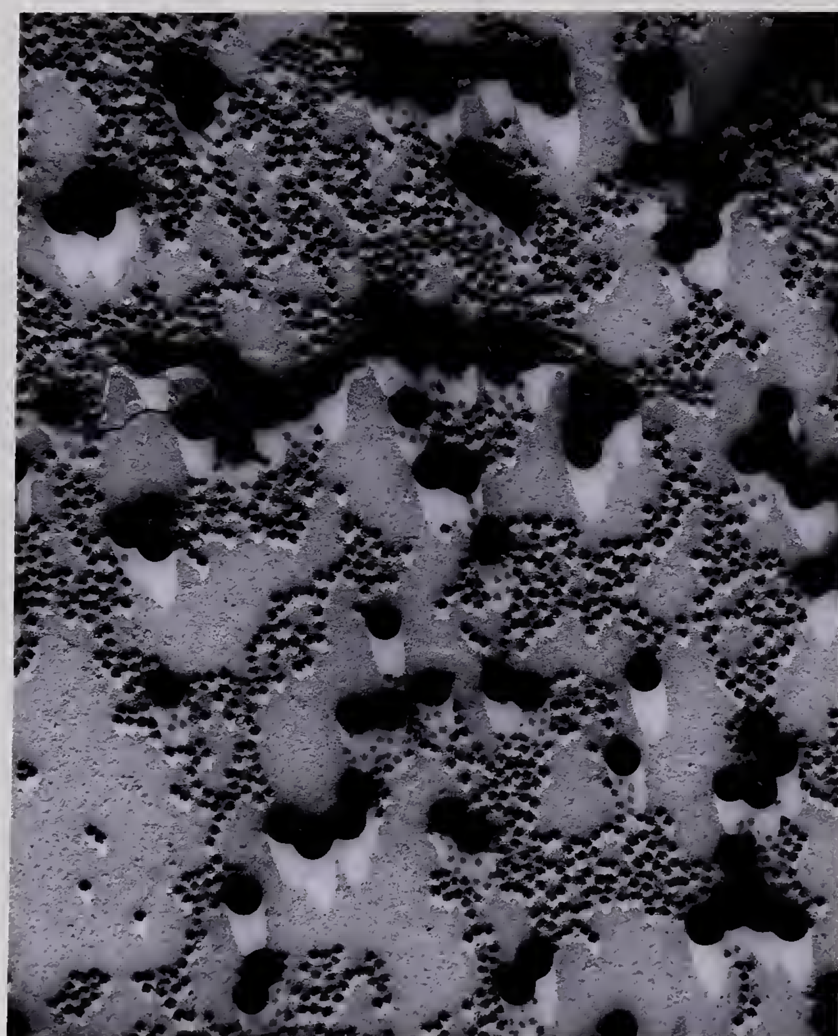


Fig. 21

ICL Virus Particle Counts

Equal volumes of purified ICL virus and 264 mμ diameter latex (3.46 x 10^{10} particles/ml) were mixed and prepared for electron microscopy by the lowered drop method of Pinteric and Taylor (1962). The preparation was shadowed with palladium. The larger dark objects are latex particles. x 16,000

TABLE IX

Virus Sample	A	B	C
Particles/ml*	4.99×10^{10}	2.74×10^{11}	6.00×10^{11}
Infectivity PFU/ml	1.0×10^{7}	5.26×10^{7}	1.1×10^{8}
HAU/ml**	8.19×10^{3}	5.24×10^{5}	1.63×10^{4}
Ratio Particles/PFU	4.99×10^{3}	5.31×10^{3}	5.5×10^{3}
Ratio Particles/HAU	6.1×10^{6}	5.23×10^{5}	3.68×10^{7}
Ratio Particles/RBC	20	1.7	121

* Average of at least eight random fields counted with each virus preparation.

** Value obtained from the correction curve for HA titrations (see Fig. 1)

The number of RBC contained in 0.25 ml of a 0.5 % suspension was counted using a Spencer bright line cell counting chamber. 0.25 ml of a 0.5 % suspension of RBC was found to contain 3.05×10^{5} RBC per ml.

The above calculations would indicate that one infectious unit (PFU) is equivalent to approximately 5,000 virus particles. The number of virus particles per HAU was not constant, ranging from 5.23×10^{5} to 3.68×10^{7}. The number of virus particles per RBC varied from 1.7 to 121.

Degradation of Intact ICL Virus

To further study the antigenic structure, the virus was degraded into its component subunits. These subunits, identified as the hexon, penton, and fibre antigens (see Fig. 22) have been described by Ginsberg *et al* (1966) for several of the human serotypes. As ICL virus has been identified as a canine adenovirus (Ditchfield *et al*, 1962; Yamamoto, 1966), there was some assurance that degradation of the virus would lead to a suspension of the three subunits.

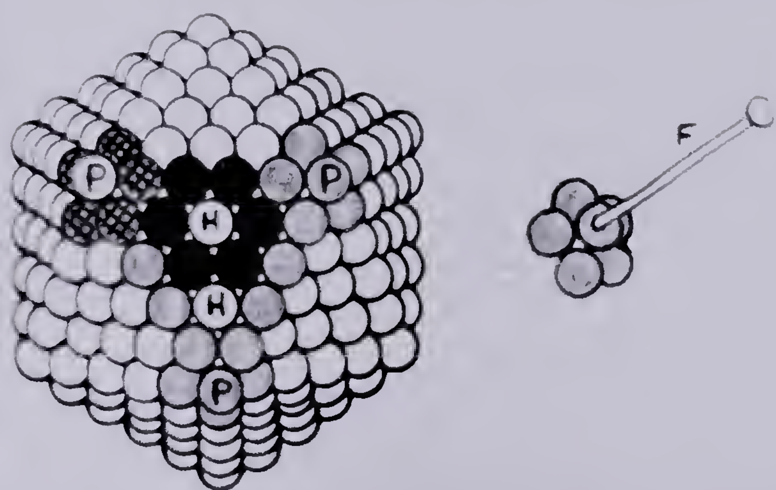

P
P
H
H
P
F

Fig. 22

<u>Schematic Representation of a Typical Adenovirus Morphology</u>

H - hexon subunit

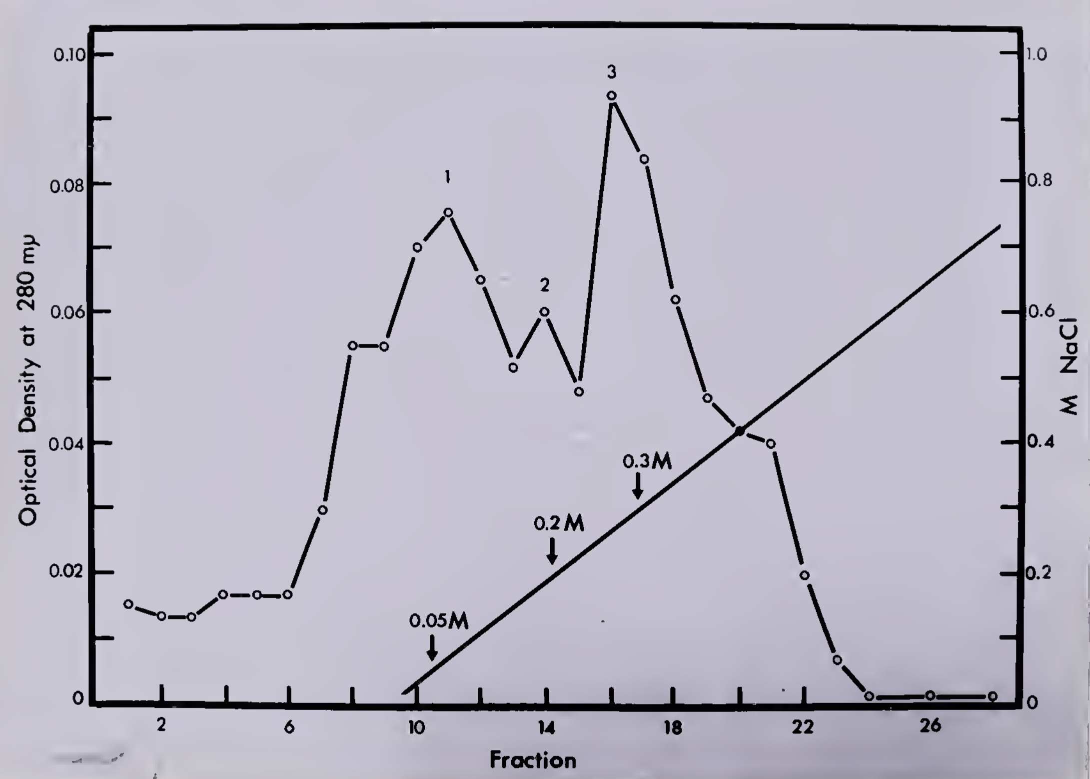
P - penton subunit

F - fibre subunit

ICL virus was degraded by incubation at room temperature for 1 hour in a solution of 0.5 % sodium lauryl sulfate (SLS), (Maizel, 1964; Smith et al, 1965). Degradation of the virus was examined with the electron microscope. In all cases, it was found that no intact virus particles were present in the degradation suspension, and only capsomere aggregates were visible.

Following degradation by the SLS method, the degraded virus suspension was dialyzed against 0.01 M Tris-HCl pH 7.2. The dialyzed suspension was then used for column chromatography studies or concentrated by dialysis against negative pressure (see Materials and Methods) and stored for immunological and electrophoretic studies.

Fig. 23 represents the following experiment: 6 ml of purified ICL virus (optical density at 280 mμ 0.67, HA titre 65,536) were degraded with SLS. After degradation the sample was assayed for HA and examined for UV absorbance at 280 mμ. The HA was completely inactivated, and the optical density was 0.55. A DEAE-cellulose column was prepared (1.3 x 20 cm) and the degraded virus suspension added and the column washed with 30 ml of buffer. The column was eluted with a gradient of NaCl in 0.01 M Tris-HCl pH 7.2. Five ml fractions were collected and each examined for UV absorbance at 280 mμ. The molarity of NaCl in each fraction was determined by conductivity measurements. Optical density measurements revealed the presence of three peaks of eluted material at 0.05, 0.2 and 0.3 M NaCl. The fractions compromising these peaks were dialyzed, concentrated and examined with the electron microscope. No structural entity could be visualized in peak 1. Peaks 2 and 3 contained individual capsomeres and structures which could not be



Optical Density at 280 mμ
0.10
0.08
0.06
0.04
0.02
0
1
2
3
0.05M
0.2 M
0.3M
M NaCl
1.0
0.8
0.6
0.4
0.2
0
2
6
10
14
18
22
26
Fraction

Fig. 23

Separation of ICL Virus Antigens on DEAE-cellulose

6 ml of purified ICL virus (optical density at 280 mμ 0.67, HA titre 65,536) were degraded with SLS. The degraded virus suspension was added to a DEAE-cellulose column (1.3 x 20 cm) and eluted with a linear gradient of NaCl in 0.01 M Tris-HCl pH 7.2.

readily identified. Fig. 24 represents a structure found in peak 2. It is composed of a central unit, surrounded by five symmetrically arranged units. This then, would be the penton antigen (less the fibre) surrounded by five hexon antigens.

Fig. 25 represents an attempt to purify the viral components by gel filtration. The filtration material was Bio-Gel A-15m (BioRad Laboratories, Richmond, California). A 1.3 x 30 cm column was prepared as outlined in Materials and Methods. The column was equilibrated with 0.01 M Tris-HCl buffer pH 7.2 and the degraded virus suspension (optical density at 280 mμ 1.18) added and washed into the column. The column was eluted with the same buffer. Three ml fractions were collected and examined for UV absorbance at 280 mμ. Only one broad peak of absorbance was obtained, indicating that there was no separation of the antigens by this technique. Fractions 5 to 9 were pooled and concentrated prior to electron microscopic examination. The electron microscopic examination revealed that capsomeres visible in the degraded virus preparation were similar in appearance before and after column chromatography.

Virus Antigen Studies by Precipitin Analysis in Agar Gel

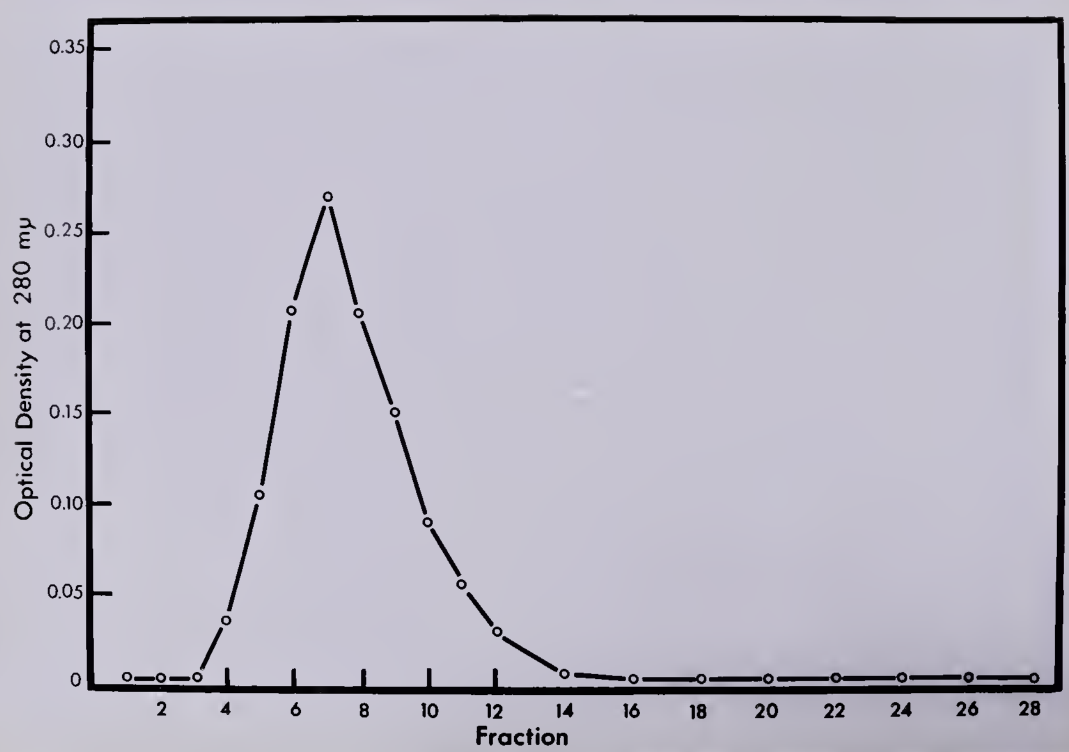
Adenovirus antigens were detected by the agar-gel diffusion precipitation test described by Ouchterlony (1949) and Pereira *et al* (1959b). The gel diffusion precipitation tests were performed in disposable plastic tissue culture plates (6 cm diameter), prepared as outlined in Materials and Methods.

Numerous experiments were carried out to determine the optimum inter-well distance, incubation temperature, incubation time, and well

Fig. 24

Penton Subunit of ICL Virus

Electron microscopic examination of material from peak 2, Fig. 23, revealed structures such as shown in the electronphotomicrograph. The central subunit is surrounded by five subunits, and is morphologically identical with the reported structure of the penton subunit. x 330,000



0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
Optical Density at 280 mμ
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Fraction

Fig. 25

Gel Filtration of Degraded ICL Virus

SLS degraded ICL virus suspension (optical density at 280 mμ 1.18) was added to a Bio-Gel A15m column (1.3 x 30 cm) and eluted with 0.01 M Tris-HCl pH 7.2. No separation of the viral subunits occurred.

diameter. Results indicated that maximum visibility of the antigen-antibody precipitin complex was attainable if the plates were prepared with a well diameter of 7 mm, an inter-well (center to center) distance of 15 mm and incubated at room temperature in a closed humidity chamber for a minimum of 48 hours. Longer incubation periods at times revealed the presence of further precipitin lines. In order to visualize these late appearing precipitates within 48 hours, the plates were immersed in a 10 % glacial acetic acid in 70 % ethanol solution to precipitate the protein complex (Parker et al, 1962).

Fig. 26 represents an experiment where the antigenic nature of ICL virus and the antigen fractions from Fig. 23 were determined. Wells were filled with approximately 0.1 ml of each of the following: 1 - partially degraded ICL virus (time degraded); 2 - aliquot from peak 1, Fig. 23; 3 - from peak 2, Fig. 23; 4 - from peak 3, Fig. 23; and 5 - calf serum. Rabbit #1950 anti-ICL virus-serum was located in the center well.

The precipitin reactions revealed that there were three distinct antigenic components in the partially degraded virus preparation. Well 2, containing material from peak 1, Fig. 23 did not appear to contain any virus antigens. However, the material may not have been detectable under the experimental conditions. Wells 3 and 4 contained virus antigens which were serologically related (as indicated by the fusion of the precipitin lines).

In order to better demonstrate the relationship of peaks 2 and 3 (Fig. 23) with a degraded virus preparation, experiments B and C (Fig. 26) were carried out. In both experiments, wells 1 and 3 contain the

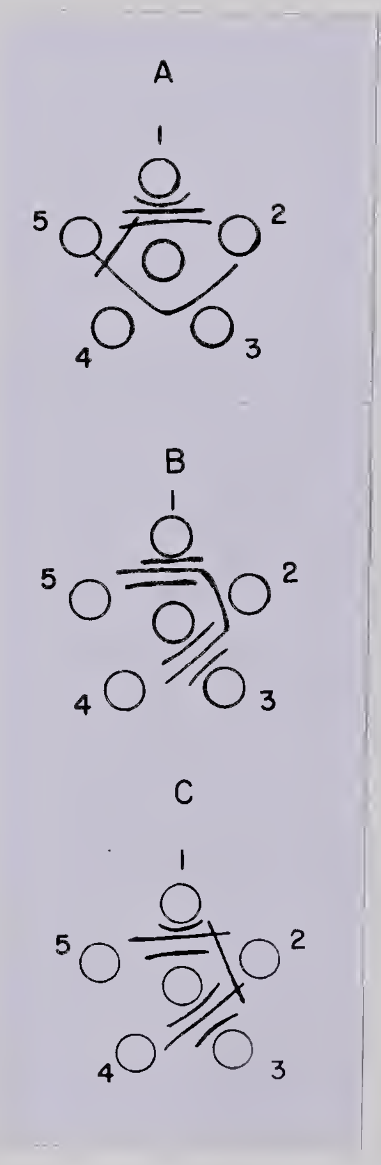

A
1
2
3
4
5
B
1
2
3
4
5
C
1
2
3
4
5

Fig. 26

Agar Gel Double Diffusion of ICL Virus Antigens

A: Well 1 - degraded ICL virus
2 - material from peak 1, Fig. 23
3 - material from peak 2, Fig. 23
4 - material from peak 3, Fig. 23

B: Wells 1 and 3 - degraded ICL virus
2 - material from peak 2
4 and 5 - blank

C: Wells 1 and 3 - degraded ICL virus
2 - material from peak 3
4 and 5 - blank

The plates were incubated at room temperature for 48 hours in a sealed humidity chamber.

degraded ICL virus, wells 4 and 5 are blank. In B, well 2 contains antigens from peak 2. Well 2, experiment C, contains material from peak 3. It is apparent that material from peak 2, is serologically related to one of the components of the degraded virus preparation. Material from peak 3, because the precipitin line was not fully extended on iehter side of the well, could not be shown to be serologically related to any of the viral components.

Viral Protein Determination (for electrophoretic studies)

The assay of HA was used throughout this study as the criterion of the relative amounts of virus present during purification. In order to know the amount of viral protein being used for electrophoretic studies, a Lowry protein determination (Lowry et al, 1951) was carried out with a suspension of purified virus. Crystalline bovine serum albumin was used as a standard, and readings were taken colorimetrically on a Beckman DB-G grating spectrophotometer at 500 mμ. A value of 108 μg of protein per ml of purified virus suspension was obtained.

Electrophoretic Studies of ICL Virus Antigens

Agar Gel Immunoelectrophoresis

Immunoelectrophoretic analysis of ICL virus was attempted according to the methods of Pereira et al (1959), Uriel (1964) and Parker et al (1962). Procedures used throughout these studies are outlined in Materials and Methods. ICL virus degraded with SLS was used.

Experimental conditions for the first experiments were as follows: Gel - 1 % agarose in 0.05 Tris-borate, pH 8.6; Electrode bath - same buffer; Power Supply - 200 volts, 1.5 mA per slide for 8 hours at room temperature; Antiserum - Rabbit #1950 anti-ICL virus-

serum. The results obtained after fixing, drying, staining, and mounting are shown in Fig. 27. Only one precipitin arc was seen with the intact virus (slide 1). No electrophoretic migration was seen to take place. The degraded virus preparation migrated towards the anode. One long precipitin arc was obtained after immunological reaction occurred (slide 2). No minor arcs could be detected. Numerous experiments carried out with the same samples and varying the voltage, amperage, and time resulted in similar results.

Agar Gel Simple Electrophoresis

Rather than continue with immunoelectrophoretic studies, it was decided to stain the viral protein after carrying out simple electrophoresis in the same agar gel used for the immunoelectrophoretic studies. The procedure used is outlined in Materials and Methods. Briefly, a well is cut in the agar gel, the sample introduced and subjected to electrophoresis under the selected conditions and for the desired time interval. First attempts using this technique proved very successful (slide 3) and the main variables such as pH, temperature, buffer concentration, voltage, and amperage were varied for optimum results. Slides 4 and 5 (Fig. 27) show the results obtained with the following system: Gel - 1 % agarose in 0.05 M Tris-borate pH 8.6; Electrode bath - same buffer; Power Supply - as indicated on each slide. Using the indicated systems, it was possible to visualize, after staining, migration of the viral protein towards the anode. However, in an attempt to achieve better separation of constituent subunits, longer time intervals of up to 12 hours were used. These longer times however, caused excessive heating which melted the agarose. Attempts to operate


1.
INTACT
pH 8.6
2.
SLS
3.
4.
5.
pH 7.0
6.
INTACT
7.
INTACT
pH 7.6
8.

Fig. 27

Immuno- and Electrophoresis in Agar Gel

The conditions for electrophoresis were as follows:

a) Immunoelectrophoresis

Slide 1: Intact ICL virus; 200 volts, 1.5 mA at pH 8.6 for 8 hours at room temperature

Slide 2: SLS degraded virus; 200 volts, 1.5 mA at pH 8.6 for 8 hours at room temperature. The vertical band in the center of the slide is an artifact formed during fixing and drying.

b) Electrophoresis

Slide 3: SLS degraded virus after gel filtration; 100 volts, 1 mA at pH 8.6 for 9 hours at room temperature

Slide 4: SLS degraded virus after gel filtration; 100 volts, 1.5 mA at pH 8.6 for 12 hours at room temperature.

Slide 5: SLS degraded virus after gel filtration, rectangular reservoir; 100 volts, 1.5 mA at pH 8.6 for 12 hours at room temperature

Slide 6: SLS degraded virus; 100 volts, 25 mA at pH 7.0 for 120 minutes at room temperature.

Slide 7: Intact ICL virus; 100 volts, 25 mA at pH 6.2 for 4 hours at room temperature

Slide 8: Intact ICL virus; 100 volts, 25 mA at pH 7.6 for 4 hours at room temperature

the apparatus at 4° C produced less migration due to the compactness of the agarose.

Electrophoretic studies carried out at pH 7.0 in 0.1 M phosphate buffer rather than 0.05 M Tris-borate pH 8.6, resulted in rapid migration of the protein. The higher amperage attainable would also increase migration of the proteins. Studies which took 6 - 7 hours at 1.5 mA per slide, were carried out within two hours at 25 mA per slide. In some cases, only one spot of stained protein was visible, but usually a faintly staining band (slide 6) close to the origin could be detected. Indications of three stained bands on several of the slides were also obtained. Experiments indicated that three distinct components with different electrophoretic mobilities were migrating towards the anode. Photographs of these slides were not obtained. Electrophoretic mobility studies at pH 6.2 and 7.6 confirmed that the viral protein was negatively charged. There was slight mobility at pH 6.2 in 0.1 M phosphate buffer compared to that obtained at pH 7.6 under the same experimental conditions (slides 7 and 8).

Starch Gel Electrophoresis

Starch gel electrophoresis was carried out using a modification of the technique described by Smithies (1955). An 11 % starch gel was prepared in 0.03 M Tris borate buffer pH 8.5. A sample of degraded virus was applied and electrophoresed for 3 hours at 150 volts and 5 mA. Electrode baths contained 0.3 M Tris-borate buffer at pH 8.5. There was no visible migration of the viral protein under these conditions.

Cellulose Acetate Electrophoresis

Two types of cellulose acetate electrophoresis studies were carried out. One, utilizing the Gelman Sepraphore cellulose acetate

strip, the other utilizing the Millipore PhoroSlide. Results in either case were negative i.e. there was no migration of virus proteins, when a sample of purified ICL virus or a sample of degraded ICL virus was used according to manufacturers instructions.

Electron Microscopic Studies

During all stages of this study, the electron microscope was utilized to determine the relative purity of ICL virus preparations. Whenever a highly purified preparation was obtained, considerable care was given to the method of negative staining employed for electron microscopic examination. Optimum staining conditions for visualization of viral subunits were obtained with dilute phosphotungstic acid (PTA) or sodium silicotungstic acid (SSTA) adjusted to pH 6.8 with KOH.

Figs. 24, 28, 29, 30 and 31 show the results obtained in several of these studies. The icosahedral structure of the ICL virus is easily seen as is the subunit assembly (shown schematically in Fig. 22). Of major interest is the presence of the fibre antigen at each vertex of the icosahedron. Fig. 31 shows a fibre antigen attached to an isolated penton subunit in a degraded virus preparation. Measurement of the fibre antigen shows that it is 35 mμ in length.

The relative size of the ICL virus was determined by mixing equal volumes of the purified virus and a suspension of 88 mμ diameter polystyrene latex particles. The preparation was negatively stained with PTA. An electronphotomicrograph is shown in Fig. 32. It is apparent that the ICL virus is slightly smaller than the 88 mμ diameter latex and would measure approximately 80 mμ in diameter.

Fig. 28

<u>Electronphotomicrograph of Intact and Partially Degraded ICL Virus</u>

The fibre subunit can be seen projecting from the vertices of the intact virus particle. The degraded virus particle appears to be slightly enlarged and partially disrupted. The preparation was negatively stained with PTA at pH 6.8. x 280,000

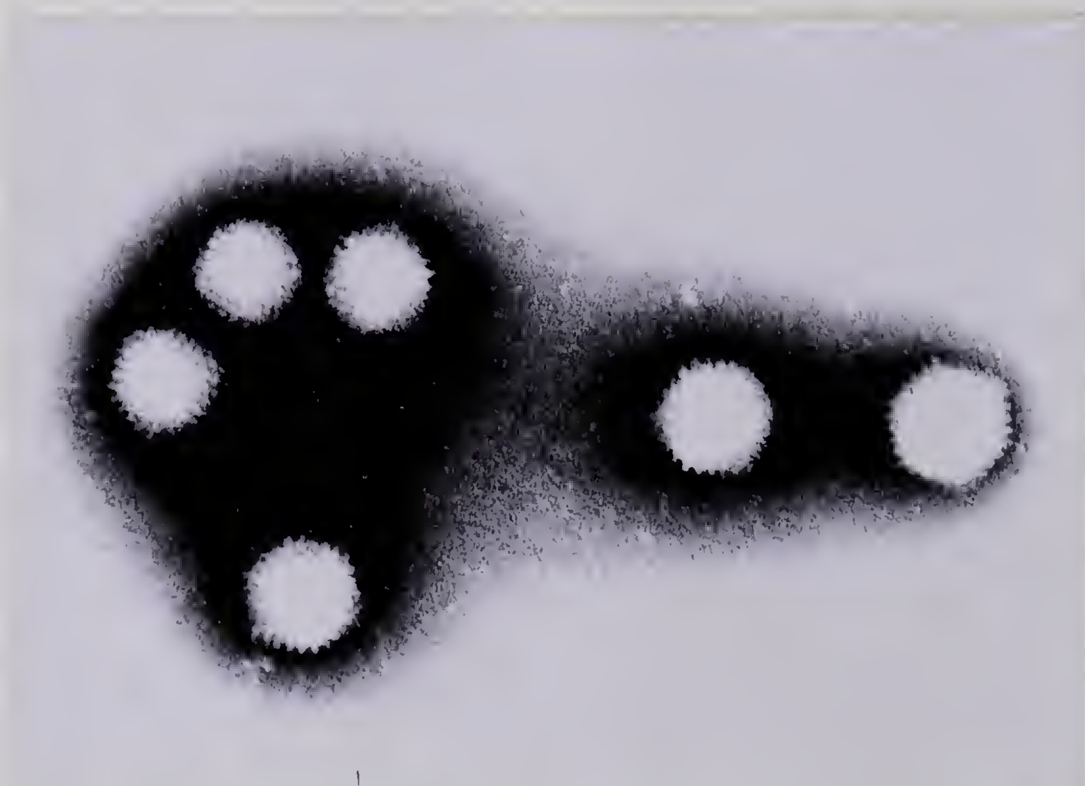


Fig. 29

Electronphotomicrograph of Intact ICL Virus

ICL virus purified by CsCl density gradient centrifugation and negatively stained with PTA at pH 6.8. Fibre subunits are easily seen projecting from several of the virus particles. x 180,000

Fig. 30

Electronphotomicrograph of Intact ICL Virus

A greater magnification of an ICL virus particle

Same conditions as Fig. 29. x 250,000

Fig. 31

Electronphotomicrograph of ICL Virus Penton and Fibre

An electronphotomicrograph of a degraded ICL virus preparation showing the isolated fibre subunit attached to a penton 'base' subunit. The preparation was negatively stained with PTA at pH 6.8. x 350,000

Fig. 32

Electronphotomicrograph of ICL Virus and 88 mμ Diameter Latex

An equal volume of 88 mμ diameter latex particles and purified ICL virus were mixed and negatively stained with PTA at pH 6.8. The electronphotomicrograph shows that ICL virus is slightly smaller than the latex particles and measures approximately 80 mμ in diameter.

DISCUSSION

ICL virus has been shown to be serologically related to the two strains of infectious canine hepatitis virus, ICH and HCC. Antisera prepared against each of the canine adenovirus strains was found to inhibit the agglutination of human 'O' RBC. Fastier (1957) reported that 'ICH virus' would agglutinate fowl RBC, but no definite proof that the virus being tested was a canine adenovirus, was given. Experiments carried out during this study have shown that ICL virus was not able to agglutinate fowl, rhesus, canine, sheep, goose, hamster, or murine RBC. However, reactions which resembled the partial agglutination of human 'O' RBC were obtained with rabbit and rat RBC. Rosen (1960) has grouped several of the human adenovirus serotypes on the basis of their ability to partially agglutinate rat RBC. In order to bring about the complete haemagglutination of the rat RBC by the human serotypes, it was necessary to add a heterologous human adenovirus serotype antiserum. Such a heterologous system is difficult to establish, as the concentrations of the virus and antiserum are very critical. A heterologous antiserum system with ICL virus was not demonstrable.

Adsorption and elution studies of ICL virus to human 'O' stroma revealed that ICL virus which could be adsorbed at 37° C to the stroma could not be eluted. Simon (1962a) has reported that human serotypes 11 and 16 could be adsorbed to rhesus RBC at 37° C and could not be eluted. He also reported on the reversible adsorption of serotypes 3, 7 and 14 at 37° C and 4° C.

Studies on the effect of trypsin on both the RBC receptor and on the ICL virus haemagglutinin revealed that only the RBC receptor was

susceptible to enzymatic attack. Kasel _et al_ (1960) described that the RDE (_Vibrio cholerae_) pretreatment of human 'O' RBC decreased the agglutinability of the RBC by certain adenoviruses. Buckland (1959) found that formalin and trypsin treatment of human 'O' RBC decreased the agglutinability of the RBC by type 9 adenovirus, in contrast to influenza virus which readily agglutinated formalized and trypsinized RBC. This phenomenon was explained by supposing that the RBC receptors for influenza and adenoviruses are different. A like supposition can be made from the experiments carried out during this study. ICL virus could not haemagglutinate trypsin treated human 'O' RBC.

Studies on the pH stability of ICL virus at room temperature have shown that the haemagglutinating property of the virus remains active over a pH range of 2.3 - 9.8 for up to 24 hours. A slight decrease in haemagglutinating titre was observed at the lower and upper limits of the pH range, but the haemagglutinating activity was not completely inactivated. The least amount of inactivation took place within the pH range of 6.3 - 9.2 and it might be supposed that there is a pH optimum for haemagglutination within these values. Yamamoto (in press) has shown that the ICL haemagglutinin is most stable at pH 5 - 6 at 56° C, but his experiments were carried out with additional cations added to the virus suspension. If an optimum pH range for ICL virus does exist, experiments carried out at 37° C might have been more informative.

Simon (1962a) has shown that the haemagglutinins of human adenovirus types 3, 7 and 14 are rapidly inactivated at pH 3 - 4 at room temperature whereas the haemagglutinins of types 11 and 16 appear to be stable at these pH values. He also found that adenovirus types 11

and 16 were stable to enzymatic attack by trypsin. ICL virus is also stable at the lower pH values, and is not sensitive to trypsin treatment. It may be that there is a relationship between pH stability and trypsin stability in certain adenovirus strains.

Yamamoto (in press) has shown the haemagglutinin of ICL to be stabilized by monovalent and divalent cations at elevated temperatures. Routinely throughout the purification procedure used in this study, ICL virus was subjected to prolonged treatment with the monovalent cation, Cs^+. An experiment carried out to study the effect of Cs^+ on the stability of the ICL virus haemagglutinin at room temperature revealed that incubation of ICL virus in 60 % CsCl for up to three weeks had no effect on the ability of the virus to haemagglutinate human 'O' RBC. In contrast, ICL virus stored for prolonged periods at room temperature in distilled water or 0.01 M Tris-HCl, rapidly lost its ability to haemagglutinate RBC. These results would tend to confirm the findings of Yamamoto that there is a cationic stabilization of the ICL virus haemagglutinin.

As Norrby (1966) and Norrby and Wadell (1967) have reported the presence of non-viral associated haemagglutinins in adenovirus type 3 and 4, it would be of interest to study the effect of cations on these entities. With both type 3 and 4 adenoviruses, the non-viral associated haemagglutinins have been shown to be composed of arrays or aggregates of the fibre antigen in a 'rosette-like' formation. As this study has shown that the haemagglutinin of ICL virus is associated with the intact virus particle, and is not separable from the virus particle, one might expect that a different cation effect may be found

for adenoviruses possessing a dissociable haemagglutinin.

Attempts to purify ICL virus using the fluorocarbon technique of Gessler et al (1956) resulted in the complete loss of all haemagglutinating activity (see Table IV). This was in contrast to the results of Norrby (1966), Valentine and Pereira (1965), and Green and Pina (1963), who successfully used fluorocarbon for adenovirus purification. Wigand et al (1966) have however, reported a similar loss of haemagglutinating activity with human serotypes 19 and 26 when a fluorocarbon purification procedure was used. Whether the fluorocarbon effects the virus was a whole or just the fibre antigen, which has been postulated to be associated with the haemagglutinating property (Wilcox and Ginsberg, 1963; Valentine and Pereira, 1965; and Norrby and Wadell, 1967), is not known.

The major problem in this study was the development of a procedure whereby the recovery of amounts of virus with a high haemagglutinating titre could be attained. Previous attempts at recovery of ICL virus from infected tissue culture harvest (Gaunt, 1966; Yamamoto, 1966) had resulted in low recoveries of haemagglutinating activity. If the postulation that the fibre antigen is the entity taking place in haemagglutination is correct, then the means by which the virus had previously been purified could have affected the integrity of the fibre. Differential centrifugation with purified ICL virus preparations by both Gaunt and Yamamoto could have resulted in breakage or removal of the fibre from the intact virus particle. If such was the case, then a milder form of virus treatment would seem justified. In this study, the CsCl cushion technique of Norrby et al (1964) was

utilized in order that the precipitating virus would not be centrifuged to a pellet but rather would be retained in a band corresponding to its buoyant density in CsCl. A broad band of virus material was obtained. Further purification of the virus by CsCl density gradient centrifugation resulted in a purified virus preparation with a high haemagglutinating titre. The three methods of preparing CsCl density gradients, (Fig. 10, 11, 12) resulted in banding of ICL virus at a buoyant density of 1.34 g/ml. This would agree with the previously reported buoyant density of 1.3388 g/ml (Gaunt, 1966).

With the recovery of virus with high haemagglutinating titres, electron microscopic studies revealed the presence of fibre-like structures at the vertices of the virus. If the intact ICL virus is composed of an icosahedron with a fibre projecting from each vertex, then, as has been shown in this study, the complete virus particle would be inactivated if the fibres were removed or damaged. Such damage or loss of fibres would account for the low recoveries of infectivity and HA in the studies of Gaunt (1966) and Yamamoto (1966). The recovery of approximately 20 % infectivity and HA with ICL virus resulted in sufficient quantities of virus for further analysis.

Attempts at utilizing DEAE-cellulose column chromatography as a method of purifying ICL virus (see Fig. 6) did not give the degree of purification desired for this study. Electron microscopic examination of the material eluting at 0.2 M NaCl revealed the presence of virus and undesirable protein.

Simon (1962b) reported the use of calcium-phosphate column chromatography as a means of purifying adenovirus types 1, 5 and 7.

Attempts made during this study to utilize this type of column were unsuccessful. ICL virus could not be adsorbed to the column and was eluted during the preliminary washing.

This study has shown that the infectivity and haemagglutinating properties of ICL virus are associated with the intact virus particle. The haemagglutinin of several of the human serotypes was shown to be separable from the intact virus particle (Zuschek, 1961; Bauer and Wigand, 1962; Bauer et al, 1964; Gelderblom et al, 1965; Norrby et al, 1964; Wigand and Wunn, 1966; Wigand et al, 1966; and Norrby, 1966). Norrby and Wadell (1967) have succeeded in showing the presence of two non-viral associated haemagglutinins in adenovirus type 4 preparations. In most of these studies, the non-virus associated haemagglutinin has been isolated by CsCl density gradient centrifugation. This study did not reveal the presence of any haemagglutinin other than that associated with the intact virus particle. Norrby (1966) was able to visualize electron microscopically that the haemagglutinin of of adenovirus type 3 was composed of a 'rosette-like' aggregation of fibre antigens. ICL virus fibre antigens were only seen associated with intact virus and occassionally in degraded virus preparations (see Figs. 29, 31).

Gaunt (1966) reported a 260:280 ratio of 1.13 for purified ICL virus. Norrby (1966) reported that the 260:280 ratio of purified adenovirus type 3 was 1.15. The value of 260:280 = 1.18 in this study agrees closely with these values.

The major problem encountered with the assay of infectivity utilizing the plaque assay technique, was the maintenance of viability of the cell monolayers. DKL cells first utilized for plaque assay of

ICL virus (Gaunt, 1966) were unsatisfactory as the cells were not viable for the duration of the incubation time required for plaque formation. Subsequent use of the MDC cell line for plaque forming ability revealed that maintenance of the agar-medium overlay pH at or about neutrality was the main factor in maintaining viability of the cells. In order to keep the pH of the overlay medium at a constant level, an additional overlay was employed on the fourth day after infection. Experiments carried out with the DKL cell line and an additional agar-medium overlay failed to maintain cell viability. The cell death might have been due to contamination of the cells with PPLO and alterations of the cell metabolism.

The addition of HLA (see Materials and Methods) to the plaque assay overlay medium had a beneficial effect on the assay system, as the number of visible plaques was increased. The addition of the HLA to the medium probably resulted in a more stringent control of the pH of the medium.

DEAE-dextran and protamine sulfate in the overlay medium of ICL infected MDC cells had a different effect than that reported in other tissue culture systems (Miles and Austin, 1963; Liebhaber and Takemoto, 1963; Colter et al, 1964). Gaunt (1966) reported that DEAE-dextran or protamine sulfate in the overlay medium of ICL infected DKL cells resulted in an increase in plaque size. Protamine sulfate in the overlay medium of ICL infected MDC cells was found to increase the number of visible plaques and markedly reduce the size of the plaques formed at concentrations greater than 50 μg per ml. DEAE-dextran had no marked effect on either the plaque size or plaque number at concentrations lower than 200 μg per ml. Colter et al (1964) and Campbell

and Colter (1965) have postulated that DEAE-dextran and protamine sulfate counteract the effect of sulfated polysaccharides that are released from the agar during autoclaving and that these inhibitors are responsible for the decrease in plaque size. However, the experiments carried out with protamine sulfate and DEAE-dextran added to the agar-medium overlay of ICL infected MDC cells did not appear to show a counteracting effect on any inhibitors that may have been present.

The addition of DMSO to the overlay medium did not appear to greatly affect the number of plaques formed, but there was a substantial increase in the size of plaques. Although the exact mechanism of action of DMSO is not known, it has been theorized that there is an increase in permeability of the cell membrane (Jacob et al, 1964; Tovell and Colter, 1967) which might allow more cells to be infected. There would, therefore, be an increase in the number of cells lysed within a given area in a given time, resulting in formation of larger, more visible plaques.

Even though the various additives had both deleterious and beneficial effects on the plaque assay system, the maintenance of proper pH was the most important factor for plaque formation.

Yamamoto (1966) reported a ratio of 100,000 to 1 virus particles to infectious unit of ICL virus. His studies however, were conducted with ICL virus purified by sedimenting of the virus to a pellet (which has been shown to be harmful to recovery of viral infectivity in this study) resulting in a marked inactivation of infectivity presumably due to breakage of the fibre antigen. In this study, using the plaque assay

method, a ratio of approximately 5,000 to 1 virus particles per infectious unit was obtained.

Particle counts carried out in three separate experiments to determine the number of virus particles per RBC in an HA assay dilution series revealed that there were from 2 to 121 virus particles per RBC. Theoretically, a 1 to 1 ratio of virus particles to RBC would result in a visible haemagglutination pattern. The data of Table IX show that by lowering the virus to RBC ratio, it might be possible to achieve this theoretical value with ICL virus.

DEAE-cellulose chromatography of SLS degraded ICL virus preparations (Fig. 23) revealed the presence of three subunits eluting at 0.05, 0.2, and 0.3 M NaCl. This was in agreement with the results of Valentine and Pereira (1965), who were able to separate the three subunits of adenovirus type 5 by DEAE-cellulose chromatography. Electron microscope examination of the ICL virus material eluting from the column, resulted in visualization of the penton and hexon structures, but not the fibre. As the size of the fibre is such that it approaches the resolving power of the electron microscope, it was not surprising that it could not be seen. Wilcox and Ginsberg (1963), who were the first to report on the presence of the fibre subunit, also had difficulty in visualizing the fibre in eluents from DEAE-cellulose columns.

Agar-gel double diffusion tests with the eluents from Fig. 23 revealed the serological relationship of the antigens. The relationship between the antigens and the components of a degraded ICL virus

preparation was determined as shown in Fig. 26. As the penton material eluting at 0.2 M NaCl was shown to be serologically related to the middle precipitin of the degraded virus preparation, it might be assumed that this precipitin was the result of a penton antigen-antibody reaction. Hexons, from peak 3, could not be shown to be related to any of the degraded virus components, as the precipitin line formed was not fully extended on either side of the well. As the hexon would be the slowest moving antigen because of its greater molecular weight, there might therefore be a serological relationship between the hexon material and the precipitin line closest to the virus reservoir. The fibre antigen, being the lowest in molecular weight, would be the fastest moving entity and serologically related to the precipitin line closest to the antiserum reservoir.

Pereira _et al_ (1959) succeeded in showing that the three antigenic components of adenovirus type 5 had different electrophoretic mobilities. The hexon and penton antigens were found to be negatively charged and moved towards the anode at pH 8.6. The fibre antigen was found to be very basic in nature as it moved towards the cathode at the same pH. Immunoelectrophoretic studies with ICL virus did not reveal three distinct antigens. Immunological reactions with anti-ICL-serum revealed only one precipitin arc (Fig. 27) on the anode side of the reservoir. Crowle (1961) states that a long precipitin arc of only moderate curvature (as obtained with the ICL preparation) suggests that it has been formed by an antigen which electrophoretically is heterogeneous and consists of a population of molecules with smoothly graduated differences in electrophoretic mobility.

If the degraded virus preparation contained an excess of hexon and penton antigens, then the subsequent electrophoretic migration of these serologically related entities would result in such an arc. Why the fibre antigen was not visible immunologically is not known. It may be that a much larger amount of the antigen is required in order to enhance staining and detection.

Agar-gel electrophoresis without the immunological reaction (Uriel, 1964) revealed that there was no basic entity in any of the degraded virus preparations studied. In certain of the electrophoretic studies carried out with a current of 25 mA (in contrast to a current of 1.5 - 3 mA normally used), there were three distinct staining proteins moving towards the anode.

Cellulose acetate electrophoresis of ICL virus preparations did not prove successful because of the large amounts of virus required. Vande Woude _et al_ (1967) have shown that in order to obtain distinctly stained bands of virus protein, virus concentrations of approximately 1 - 8 mg per ml were necessary. Such a quantity of ICL virus was not available.

Electron microscopic studies of ICL virus have now well established that the virus is an icosahedron (Yamamoto and Marusyk, in preparation) of 252 capsomeres. The observations carried out during this study have shown that ICL virus has a fibre antigen at each vertex of the icosahedron. Observation of degraded virus preparations revealed attachment of the fibre and penton antigens (Fig. 31) as reported for the human adenovirus types 3 and 5 (Valentine and Pereira, 1965; Norrby, 1966).

The fibre subunit of ICL virus was found to be 35 mμ in length. Valentine and Pereira (1965) found the fibre subunit of adenovirus type 5 to be 24 mμ in length while Norrby (1966) found that of adenovirus type 3 to be 8 - 11 mμ long. Further studies of other adenoviruses might reveal that the differing lengths of the fibre subunit may involve differing infectious and haemagglutinating characteristics.

The structure of negatively stained virus particles compared to that of sectioned particles appears to have certain differences. In electrophotomicrographs of sectioned virus particles, it is possible to visualize a narrow region between the protein shell and the internal DNA (Yamamoto, personal communication) which is not visible in negatively stained preparations. The relationship of this area to the antigenic nature of the virus is unknown.

-- Nothing brings us so close
to the riddle of life -
and to its solution -
as viruses --

W. Weidel, 1959

BIBLIOGRAPHY

Allison, A., Pereira, H., and Farthing, C. 1960. Investigation of Adenovirus Antigens by Agar Gel Diffusion Techniques. Virology 10: 316.

Araki, C. 1959. Seaweed Polysaccharides. Proc. 4th Inter. Congr. Biochem. Vienna, 1958. 1: 15. MacMillan (Pergamon). New York.

Bauer, H., and Wigand, R. 1962. Heterogeneity of Adenovirus Haemagglutinins. Archiv. ges. Virusforschung. 12: 148.

Bauer, H., Wigand, R., and Adam, W. 1964. Losliche und Virusgebundene Adenovirus Hamagglutinine. Z. Naturforschung. 19b: 587.

Bonifas, V., and Schlesinger, R. 1959. Nutritional Requirements for Plaque Production by Adenoviruses. Federation Proc. 18: 560.

Buckland, F. 1959. Impairment of Viral Haemagglutination of Red Cells after Treatment with Formalin. Nature 183: 1276.

Buckland, F., and Tyrell, D. 1963. A Comparative Study of Virus Hemagglutinins. The Stability of Hemagglutinins and Red Cell Receptors to Certain Physical and Chemical Treatments. J. Gen. Microbiol. 32: 241.

Campbell, J. B., and Colter, J. S. 1965. Studies of Three Variants of Mengo Encephalomyelitis Virus II. Inhibition of Interaction with L-cells by an Agar Inhibitor and by Protamine. Virology 24: 578.

Crowle, A. 1961. In: Immunodiffusion, p. 87. Academic Press. New York.

Dulbecco, R. 1952. Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus. Proc. Natl. Acad. Sci. (US) 38: 747.

Dulbecco, R., and Vogt, M. 1954. Plaque Formation and Isolation of Pure Lines with Poliomyelitis Virus. J. Exptl. Med. 99: 167.

Ditchfield, J., MacPherson, L., and Zbitnew, A. 1962. Association of a Canine Adenovirus (Toronto A26/61) with an Outbreak of Laryngotracheitis (Kennel Cough). Can. Vet. J. 3: 238.

Espmark, J., and Salenstedt, C. 1961. Hemagglutination-inhibition Test for Titration of Antibodies Against *Hepatitis contagiosa canis* (Infectious Canine Hepatitis). Archiv. ges. Virusforschung. 11: 64.

Fastier, L. 1957. Studies on the Hemagglutinin of Infectious Canine Hepatitis Virus. J. Immunol. 78: 413.

Flanagan, J., and Ginsberg, H. 1962. Synthesis of Virus-specific Polymers in Adenovirus-infected Cells: Effect of 5-fluoro-deoxyuridine. J. Exptl. Med. 116: 141.

Friedman, M., and Bennett, C. 1957. A Hemagglutination Test for Detection of Adenovirus Antibodies. Proc. Soc. Exptl. Biol. & Med. 94: 712.

Gaunt, R. 1966. MSc. Thesis. Department of Microbiology, University of Alberta.

Gelderblom, H., Wigand, R., and Bauer, H. 1965. DEAE-Chromatography of Adenoviruses of Rosen's Group II. Nature 205: 625.

Gessler, A., Bender, C., and Parkinson, M. 1956. A New Rapid Method for Isolating Viruses by Selective Fluorocarbon Deproteinization. Trans. N.Y. Acad. Sci. 18: 701.

Ginsberg, H., Pereira, H., Valentine, R., and Wilcox, W. 1966. A Proposed Terminology for the Adenovirus Antigens and Virion Morphological Subunits. Virology 28: 782.

Gomori, G. 1955. In: Colowick, S., and Kaplan, N. ed. Methods in Enzymology, Vol. I. p. 138. Academic Press. New York.

Green, M., and Pina, M. 1963. Biochemical Studies on Adenovirus Multiplication IV Isolation, Purification, Chemical Analysis of Adenovirus. Virology 20: 199.

Greene, A., Silver, R., Krug, M., and Coriell, L. 1965. A Premixed Powder for Preparation of Tissue Culture Media. Proc. Soc. Exptl. Biol. & Med. 118: 122.

Hanks, J., and Wallace, R. 1949. Relation of Oxygen and Temperature in the Preservation of Tissue by Refrigeration. Proc. Soc. Exptl. Biol. & Med. 71: 196.

Horne, R., Brenner, S., Waterson, A., and Wildy, P. 1959. The Icosahedral Form of an Adenovirus. J. Mol. Biol. 1: 84.

Jacob, S., Bischel, M., and Herscher, R. 1964. DMSO: Effects on the Permeability of Biologic Membranes. Current Therap. Res. 6: 134.

Kalter, S. 1963. Procedures for the Routine Laboratory Diagnosis of Viral and Rickettsial Infections. Burgess Publishing Co. New York.

Kasel, J., Rowe, W., and Nemes, J. 1960. Modification of Erythrocyte Receptors by a Factor in Adenovirus Suspension. Virology 10: 388.

Kjellen, L. 1961. A Study of the Adenovirus-host Cell System by Plaque Assay. Virology 14: 234.

Klemperer, H., and Pereira, H. 1959. Study of Adenovirus Antigens Fractionated by Chromatography on DEAE-cellulose. Virology 9: 536.

Kunishige, S., and Hirato, K. 1960. Immunological Studies on the Infectious Hepatitis Virus II. Hemagglutinin. Jap. J. Vet. Res. 8: 271.

Liebhaber, H., and Takemoto, K. 1961. Alteration of Plaque Morphology of EMC Virus with Polycations. Virology 14: 502.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. 1951. Protein Measurement with the Folin Reagent. J. Biol. Chem. 193: 265.

Maizel, J. 1964. Studies on the Structural Proteins of Adenovirus. Bacteriol. Proc. Abstr. 64th Ann. Meeting Am. Soc. Microbiol. p. 125.

Meselson, M., Stahl, F., and Vinograd, J. 1957. Equilibrium Sedimentation of Macromolecules in Density Gradients. Proc. Natl. Acad. Sci. (US) 43: 581.

Miles, J., and Austin, F. 1963. The Formation of Plaques in Tissue Culture by Arboviruses. Austral. J. Exptl. Med. 41: 199.

Norrby, E. 1966. The Relationship Between the Soluble Antigens and the Virion of Adenovirus Type 3 I. Morphological Characteristics. Virology 28: 236.

Norrby, E., Gronberg, M., and Magnusson, P. 1964. Virusspecific Products Appearing During the Multiplication of Adenovirus Type 3. Unio Nordica Contra Cancrum, Symp. Virus and Cancer p. 118.

Norrby, E., and Wadell, G. 1967. Soluble Components of Adenovirus Type 4. Virology 31: 592.

Ouchterlony, O. 1949. Antigen-Antibody Reactions in Gels. Acta. Path. Microbiol. Scand. 6: 507.

Parker, W., Starkiw, W., and Wilt, J. 1962. C-Reactive Protein in Virus Infection. Can. Med. Assoc. J. 87: 791.

Pereira, H., Allison, A., and Farthing, C. 1959a. Study of Adenovirus Antigens by Immunoelectrophoresis. Nature 183: 896.

Pereira, H., Pereira, M., and Allison, A. 1959b. Use of Gel Diffusion Precipitation Test in the Diagnosis of Adenovirus Infections. Lancet I: 551.

Pereira, H., Huebner, R., Ginsberg, H., and Van der Veen, J. 1963. A Short Description of the Adenovirus Group. Virology 20: 613.

Pereira, H., Pereira, M., and Clarke, S. 1965. Human Adenovirus Type 31. A New Serotype with Oncogenic Properties. Lancet i: 21.

Peterson, E., and Sober, H. 1962. In: Colowick, S., and Kaplan, N. ed. Methods in Enzymology, Vol. V. p. 3. Academic Press. New York.

Pinteric, L., and Taylor, J. 1962. The Lowered Drop Method for the Preparation of Specimens of Partially Purified Virus Lysates for Quantitative Electron Microscopic Analysis. Virology 18: 359.

Reed, L., and Muench, H. 1938. A Simple Method of Estimating 50 % End Point. Am. J. Hyg. 27: 493.

Rosen, L. 1958. Hemagglutination by Adenoviruses. Virology 5: 574.

Rosen, L. 1960. A Hemagglutination-inhibition Technique for Typing Adenoviruses. Am. J. Hyg. 71: 120.

Rosen, L., Hoves, J., and Bell, J. 1962. Further Observations on Typing Adenoviruses and a Description of Two Possible Additional Serotypes. Proc. Soc. Exptl. Biol. & Med. 110: 710.

Rouse, H., Bonifas, V., and Schlesinger, R. 1963. Dependence of Adenovirus Replication on Arginine and Inhibition of Plaque Formation by Pleuro-pneumonia Like Organisms. Virology 20: 357.

Rowe, W., Huebner, R., Gilmore, L., Parrot, R., and Ward, T. 1953. Isolation of a Cytopathic Agent from Human Adenoids Undergoing Spontaneous Degeneration in Tissue Culture. Proc. Soc. Exptl. Biol. & Med. 84: 570.

Simon, M. 1962a. Haemagglutination Experiments with Certain Adenovirus Type Strains. Acta. Microbiol. Hung. 9: 45.

Simon, M. 1962b. Chromatography of Adenoviruses on Calcium-Phosphate Columns. Acta. Virologica 6: 302.

Smith, K., Gehle, W., and Trousdale, M. 1965. Architecture of the Adenovirus Capsid. J. Bacteriol. 90: 254.

Smithies, O. 1955. Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults. Biochem. J. 61: 629.

Spiegel, M. 1961. Theory and Problems of Statistics. Schaum Publishing Co. New York.

Takemoto, K., and Liebhaber, H., 1961. Virus-polysaccharide Interactions. I. An. Agar polysaccharide-Determining Plaque Morphology of EMC Virus. Virology 14: 456.

Tovell, D., and Colter, J. 1967. Observations on the Assay of Infectious Viral RNA: Effects of DMSO and DEAE-Dextran. Virology 32: 84.

Uriel, J. 1964. In: Grabar, P., and Burton, P. ed. Immunoelectrophoretic Analysis, p. 30. Elsevier Publishing Co. New York.

Valentine, R., and Pereira, H. 1965. Antigens and Structure of the Adenovirus. J. Mol. Biol. 13: 13.

Vande Woude, G., Trautmann, R., and Bachrach, H. 1967. The Influence of the Host Cell, Ionic Strength and Chymotrypsin on Foot-and-Mouth Disease Virus Electrophoretic Mobility. Archiv. ges. Virusforschung. 20: 71.

Wigand, R., and Wunn, W. 1966. Virus Associated and Soluble Adenovirus Hemagglutinins: Differences in Antigenic Specificity. Virology 28: 484.

Wigand, R., Wunn, W., Schmidt, W., and Gelderblom, H. 1966. Verfahren zur Trennung von Adenovirus-Antigenen. Archiv. ges. Virusforschung. 19: 319.

Wilcox, W., and Ginsberg, H. 1961. Purification and Immunological Characterization of Type 4 and 5 Adenovirus Soluble Antigens. Proc. Natl. Acad. Sci. (US) 47: 512.

Wilcox, W., and Ginsberg, H. 1963. Structure of Type 5 Adenovirus I. Antigenic Relationship of Virus-Structural Proteins to Virus-Specific Soluble Antigens from Infected Cells. J. Exptl. Med. 118: 295.

Yamamoto, T. 1963. Multiplication and Morphology of a Recently Isolated Canine Adenovirus. Bacteriol. Proc. Abstr. 63rd Ann. Meeting Am. Soc. Microbiol. p. 120.

Yamamoto, T. 1966. Some Physical and Growth Characteristics of a Canine Adenovirus Isolated from Dogs with Laryngotracheitis. Can. J. Microbiol. 12: 303.

Yamamoto, T. 1967. Effect of Ion Concentration and pH on the Thermal Stability of a Canine Adenovirus. Can. J. Microbiol. in press.

Zuschek, F. 1961. Studies of the Hemagglutinins of Type 3, 4, and 7 Adenovirus. Proc. Soc. Exptl. Biol. Med. 107: 27.